Megalencephalic Leukoencephalopathy with Subcortical Cysts
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Summary, Discussion, and Prospects
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1. Clinical Description

The studies described in this thesis all started when a ten-year-old boy with macrocephaly and cerebellar ataxia visited the out-patient department of the VU University Medical Center in 1991. MRI analysis revealed that this boy had a disease involving the white matter of the brain. Soon after this patient other patients with strikingly similar clinical and MRI features were detected, as reported by Van der Knaap et al. Since then several other groups have described similar cases. In none of these children a specific diagnosis could be established or a basic biochemical defect could be identified. The homogeneous clinical picture and MRI findings were suggestive of a ‘new’ disease entity, now known as ‘Megalencephalic Leukoencephalopathy with subcortical Cysts’ (MLC). Characteristic clinical features of the disease are a macrocephaly that arises in the course of the first year of life and a delayed onset of motor deterioration, dominated by cerebellar ataxia. Characteristic MRI features include diffuse cerebral white matter signal abnormalities, swelling of the affected white matter, and the typical subcortical cysts.

2. Discovery of the MLC1 gene

The high frequency of consanguinity of the parents and occurrence of families with two affected siblings suggested an autosomal recessive mode of inheritance. In the late nineties we had collected enough informative families (four consanguineous families and one family with two affected children) to start a genetic linkage study with microsatellite markers. In the mean time, a Turkish-French consortium detected the locus for the disease gene at the very end of the long arm of chromosome 22 (22qtel). The critical region, however, was too large to identify the disease gene. We used the information of our families to limit the critical region to a DNA stretch that only contained four genes. Mutation analysis showed mutations in the second gene investigated, KIAA0027, which we renamed into MLC1. The discovery of this gene is described in chapter 2.

3. Characterization of the MLC1 gene

3a. Types of mutations

Over the years many different mutations in the MLC1 gene have been found. These mutations are distributed along the whole gene and include all different types: splice-site mutations, nonsense mutations, missense mutations, deletions, and insertions.
Novel mutations in our patient population are described in chapter 3. In chapter 4 an update is given describing all mutations found up to 2005. Although most families have unique mutations, evidence for a founder effect is present in four communities 13,16,18,20. The strongest evidence for a founder effect is found in the Indian Agarwal community 13,20. All patients within this community share the same insertion (c.135_136insC) causing a frameshift (p.Cys46LeufsX34) and the appearance of a premature stop.

3b. Extended mutation analysis
In about 20% of the typical MLC cases, diagnosed by clinical and MRI criteria, no or only one MLC1 mutation can be found. One reason for not finding mutation(s) is the standard mutation analysis at genomic level, which may miss heterozygous deletions, mutations in the promoter or 3’- and 5’-untranslated regions, and intron mutations that may affect splicing of the mRNA. For this reason, we performed extended mutation analysis in patients that have only one or no mutations in the MLC1 gene. This analysis included sequencing of cDNA derived from lymphoblasts of the patients to show any missed splice-site mutations, and qPCR to elucidate differences in expression levels of the MLC1 gene. Chapter 4 provides, in addition to the mutation update, information on mutations found with the extended mutation analysis.

3c. Evidence for additional genes involved in MLC
Several families, in which no mutations are found, also do not show linkage with the MLC1 locus. This indicates that there must be more than one gene associated with MLC 14,21,23. Therefore linkage analysis was performed in four informative families, without mutations in the MLC1 gene and without linkage on the MLC1 locus, which resulted in several candidate regions. Some promising genes in these regions were sequenced, but unfortunately no mutations have been found. Since there are too many candidate regions, this study will only be continued when DNA from additional informative families will become available that can possibly reduce this number. It should be noted that the identification of multiple candidate regions may not only be due to the limited number of informative families, but can also be the result of several additional genes being involved in MLC.

The consequence of the above findings is that the diagnosis remains primarily based on clinical and MRI findings. Thus, if the MRI shows the typical features of MLC in an ataxic patient with macrocephaly, the diagnosis is MLC even if no mutations are found in the MLC1 gene.
4. Genotype-phenotype correlation

No genotype-phenotype correlation has been observed. So, the severity of the phenotype does not appear to correlate with the type and position of the mutation(s) in the gene. There is no phenotypic difference between MLC patients with mutations in MLC1 and patients without. Patients from the Agarwal community, who are all homozygous for the same mutation, exhibit a wide phenotypic variation.

5. Localization of the MLC1 protein

The MLC1 gene encodes a 377 amino acid plasma membrane protein, called MLC1. In chapter 5 we report that this protein contains eight transmembrane domains and that it is highly conserved throughout evolution in a variety of myelin-producing vertebrates (see Figure 1). Moreover we show that, in the brain, MLC1 is expressed in distal processes of astrocytes at the blood-brain and CSF-brain barriers and in the cellular processes of Bergmann glia. This specific localization of MLC1 strongly suggests that MLC1 is localized in astroglial endfeet, but higher-resolution electron microscopic (EM) studies are necessary to show the exact localization. In support of this suggestion, Teijdoo et al. already showed that mouse MLC1 is expressed in astrocytic endfeet. Besides the expression in brain, MLC1 is also expressed in all types of leukocytes, as shown in chapter 5. The significance of this expression remains unclear. There is no evidence that mutations in the MLC1 gene cause a defect in leukocyte function in patients.

Recently, Teijido et al. published a paper about the expression of MLC1 in mice. They showed that MLC1 is expressed in neurons in the adult mouse brain. In addition, EM studies showed a more precise localization of MLC1 in the plasma membrane and in vesicular structures of neurons. In the developing brain, MLC1 was mainly expressed in axonal tracts during early stages of development. So far, we have not been able to confirm these results in humans. We, however, did not immuno-stain the complete human brain systematically yet. Another contradiction with the human situation is MLC1 expression in the peripheral nervous system. The lack of expression in humans is in line with the absence of ‘peripheral dysfunction’ in MLC patients. Teijdoo et al. also described EM studies showing that MLC1 is localized in astrocyte-astrocyte junctions, and not in the perivascular membrane. They therefore concluded that MLC1 can not be a part of the dystrophin-associated glycoprotein complex (DGC) (see chapter 6 for further discussion). To confirm or reject these findings between species it is necessary to perform EM-studies in the human brain.
It is important to note that MLC is the second genetic defect that specifically involves an astrocytic protein. The first genetic defect was described in 2001 by Brenner et al. who demonstrated that Alexander disease is caused by mutations in GFAP, a cytoskeleton protein that is only present in astrocytes. Interestingly, both MLC and Alexander disease are childhood leukoencephalopathies.

**Figure 1:** Alignment of the protein products of the MLC1 genes of several vertebrates. Gray block Conservative differences black blocks highly conserved. The eight predicted transmembrane domains are boxed.

### 6. Binding partners of MLC1

A multi-subunit complex called the dystrophin-glycoprotein complex (DGC) is expressed in astrocytic endfeet. This complex is expressed in different tissues and has been characterized best in skeletal muscle where it connects the cytoskeleton of a muscle fiber to its surrounding extracellular matrix. Mutations in different components of the DGC disrupt this complex and lead to various muscular dystrophies. In the congenital muscular...
dystrophies (CMDs), the muscular phenotype is often combined with brain abnormalities, including white matter abnormalities.

From early on, a striking similarity in MRI features of MLC and CMD with merosin deficiency (MDC1A) was noted. MDC1A is caused by mutations in the LAMA2 gene, encoding the laminin-α2 chain of merosin, a member of the DGC. Patients with MDC1A also have diffusely abnormal, mildly swollen cerebral white matter and in some cases there are anterior temporal cysts. In addition, microscopic examination of the brain revealed myelin vacuolation in MDC1A, similar to MLC. The striking similarities in MRI features and pathology between MLC and MDC1A, together with the specific localization of both MLC1 and merosin at astrocytic endfeet, led to the hypothesis that the MLC1 protein might be associated with the DGC in the brain (chapter 6).

To test this hypothesis, a general feature of the DGC was used: if one of its composing proteins is mutant or missing, the complex can be instable and other DGC-members can show reduced or altered expression. We, therefore, tested the (co-) localization of MLC1 and several DGC-members in control, glioblastoma and MLC brain tissue. The results in chapter 6 show an almost perfect co-localization of MLC1 and members of the DGC. Altered expression of MLC1 and aquaporin-4 was found in glioblastoma tissue. Additionally, the absence of MLC1 and altered expression of both agrin, Kir4.1 and α-dystroglycan in brain tissue of MLC patients was demonstrated. Furthermore, a direct protein interaction between MLC1 and Kir4.1 was shown. Unfortunately, we were not able to immunoprecipitate multiple DGC-members with MLC1 antibodies, despite great efforts of two laboratories (Amsterdam and Barcelona). This might be due to the inability of these antibodies to immunoprecipitate or because immunoprecipitation is not possible between these proteins. Together the above findings provide strong evidence for an association between MLC1 and DGC-members. Ambrosini et al. recently provided additional evidence for functional and structural relationships between MLC1 and the DGC.

Virtually all known defects in DGC-proteins have been associated with a muscular dystrophy, which is absent in MLC patients. This may be due to a difference in DGC composition for muscle compared to brain tissue. Whereas merosin is expressed both in muscle and brain, MLC1 is not expressed in skeletal muscle.

Another striking similarity between MLC and MDC1A is in that both diseases the water content of the affected white matter is abnormally high due to intramyelinic vacuole formation. The DGC is crucial for anchoring of water and potassium channels at the perivascular endfeet. Disruption of the DGC may lead to altered ion and water homeostasis of the brain and result in an increased water content within myelin.
7. Functional studies on the MLC1 protein

Several observations prompted us to state the hypothesis that MLC1 has a transporter or channel function. First of all, amino acid sequence analysis reveals a weak similarity between MLC1 and potassium channel Kv1.1, ABC-2 type transporters, and sodium:galactoside symporters. Secondly, MLC1 contains an internal repeat, found in several ion channel proteins. Based on these theoretical findings, several papers have suggested a possible transporter function of the MLC1 protein. Thirdly, as described in chapter 5, MLC1 has eight transmembrane domains. At the time, the Swissprot database contained 22 human proteins with eight transmembrane domains of which the majority (17 proteins) has a transporter or channel function.

To test this hypothesis we applied the whole-cell patch clamp technique and demonstrated that the MLC1 expression-induced current is carried by chloride. In chapter 7 we also described that this chloride transport is associated with volume regulation. The chloride channel activity shows similarities with the activity of the so-called volume-regulated anion channels (VRACs). The modulation of this VRAC has been hypothesized to influence astrocytic cell volume and ion homeostasis in the brain. Although the biophysical characteristics of VRACs have been investigated extensively, the related proteins have not been identified. Specific criteria for VRAC activity have been published: (I) VRAC is activated by hypo-osmotic shock and associated cell swelling; (II) currents through VRAC are outwardly rectifying and display only time-dependent inactivation at high positive potentials; (III) VRAC currents are mainly carried by chloride; (IV) VRAC is inhibited by anti-estrogens (for example Tamoxifen); and (V) VRAC activation requires intracellular ATP. In our study we show chloride channel activity associated with the presence of wild-type MLC1. Based on these results we conclude that MLC1 is either a chloride channel involved in volume regulation or a protein indispensable for the function of such channel. To elucidate the latter issue, we studied Sf9 insect cells, which are evolutionary very distant from human cells. They do not contain a MLC1 orthologue and therefore most likely also lack the natural binding partners of MLC1. The finding that MLC1 expression induced a chloride current profile associated with volume regulation in Sf9 cells strongly suggests that MLC1 harbors a channel function itself. Interestingly, recently Blanz et al. published a paper about chloride channel CLC-2 knock-out mice, that show brain pathology that is highly similar to MLC brain pathology. This is another hint that MLC1 may indeed be involved in chloride transport.

MLC patient-derived lymphoblasts lack the chloride current profile as well as cell swelling under hypo-osmotic conditions. Strikingly, the lack of cell swelling under hypo-osmotic conditions is also seen in control lymphoblasts, when chloride channel function is
inactivated by Tamoxifen. Hence, water influx and the activity of this MLC1-related chloride channel must either be mutually dependent, co-regulated, or use the same channel in these cells. In astrocytes a functional interdependence between MLC1 and a water channel could involve the water channel Aquaporin-4 (AQP4), which is, like MLC1, localized in astrocytic endfeet \(^{34,47}\). In addition, both MLC1 and AQP4 are associated with the DGC (chapter 6). Interestingly, Benfenati et al. demonstrated that knockdown of AQP4 in rat astrocytes leads to a decrease of VRAC-mediated currents \(^{48}\), supporting such functional interdependence. In addition, Amiry-Moghaddam et al. showed an example of functional interdependence between AQP4 and another ion channel (Kir4.1) in astrocytic endfeet, which is also part of the DGC. Disruption of the AQP4 localization severely affected the Kir4.1 function, but its localization was unchanged \(^{49}\). So far, we have been unable to confirm a physical interaction between MLC1 and AQP4 despite extensive immunoprecipitation studies (data not shown). Alternatively, one may consider the possibility that activated chloride channels themselves are able to transport water \(^{50}\). Further detailed studies are required to unravel the relationship between this MLC1-associated chloride channel and water homeostasis.

The complete absence of chloride currents in cells from patients with a frame-shift mutation is indicative of a non-functional channel in these patients. Similar findings, however, were obtained in cells from a MLC patient with missense mutations. Absence of function cannot be solely explained by a complete absence of MLC1 in the plasma membrane, because Teijido et al. \(^{24}\) showed that a significant amount of MLC1 with amino acid substitutions is expressed in the plasma membrane. Amino acid changes probably affect the conformation of the protein and abolish its function.

In chapter 7 evidence was provided that increased white matter water content and vacuolation of myelin and astrocytic endfeet are the cellular consequences of MLC1 dysfunction. In chapter 5 it was reported that MLC1 is almost exclusively present in leukocytes and brain tissue. There is, however, no evidence for abnormal leukocyte morphology or function. In addition, it was shown in chapter 7 that MLC1 expression is much higher in brain than in leukocytes, indicating that the implications of our electrophysiological findings are much more important for astrocytes than for leukocytes and negligible for other cell types. The differential expression of MLC1 would explain the exclusive involvement of the cerebral white matter in MLC. Chloride channel activity associated with cell swelling has been documented in many different cell types. This activity in cells other than astrocytes is probably attributable to proteins other than MLC1. In line with this, VRAC kinetics and pharmacology have been shown to be different for different tissues, indicative of the molecular diversity of VRAC \(^{45}\).

In summary, our data suggest that MLC1 with a normal function is an absolute prerequisite for chloride channel activity associated with cell swelling, given the total lack of hypo-
osmotically induced chloride currents in MLC patient-derived cells. The ultimate test
to show whether MLC1 is a volume-regulated chloride channel is to isolate the MLC1
protein in a lipid bilayer, containing no other proteins than MLC1, and to record the
response to whole-cell chloride currents in both iso- and hypotonic conditions, or to
show anion-selectivity changes by using mutant MLC1 constructs. It might, however,
be difficult to find the amino acid changes that are important for pore properties and
gating. Alternatively, MLC1 may be a component of a complex in which another channel
protein has chloride channel activity. In astrocytes, proper MLC1 function would then be
an absolute requirement for activity of this complex.

8. Remaining questions

One of the most important remaining question of this thesis is how dysfunction of chloride
channel activity, associated with volume regulation in astrocytes, leads to inclusion of
vacuoles in the outer layers of the myelin sheaths and in astrocytic endfeet. This question
is not easy to answer. Patient data indicate that the head circumference and neurological
condition are normal at birth. The head circumference increases rapidly over the first year
of life and by 9-12 months all patients have a prominent macrocephaly. At birth, the brain
contains hardly any myelin and brain development during the first year of life is dominated
by rapid myelin deposition in the white matter. This suggests that the deposited myelin
contains countless vacuoles from the beginning, explaining that the megalencephaly and
associated macrocephaly arise at the same time as the myelin is deposited.

During this process of myelination, substantial amounts of water need to be removed
for a proper compact myelin sheath formation. Kamasawa et al. 2005 suggests two
pathways to transport excess ions and water to the external layers of the myelin sheath:
(I) cytoplasmic diffusion via the cytoplasmic loops of myelin at paranodes and Schmidt-
Lanterman incisures, or (II) more direct radial diffusion to the outer layer by interlamellar
connexin32-containing gap junctions. From the outer lamellae of the myelin sheath,
ions and water are transported through oligodendrocyte-to-astrocyte gap junctions
(containing both connexin32 and connexin46.6 (same as connexin47 in mice)) into the
astrocytic syncytium and then passed on to astrocytic endfeet at capillaries and the
glia limitans 51. Insufficient clearance of water and ions due to dysfunctional chloride
channel activity may lead to inclusion of vacuoles in myelin sheaths during deposition.
Once trapped, the vacuoles may be permanent. The likelihood of this interpretation is
substantiated by the Cx32/Cx47 double knockout mouse that displays vacuoles in the
innermost and outermost myelin layers 52.
9. Possible treatment for MLC patients

The phenomenon that MLC patient-derived lymphoblasts are not able to swell under hypotonic conditions can be used in the search for treatment of MLC. By high throughput screening of large libraries of chemicals, the ability of individual chemicals to increase cell swelling in patient-derived lymphoblasts can be tested. The ideal is to find a molecule that is specific and will not interfere with other, related targets. Recently Welch et al. published a paper about a new drug with potential therapeutic options for patients with nonsense mutations. In this paper a new chemical entity, PTC124, was described that selectively induces ribosomal read-through of premature terminations codons by performing throughput screenings. Table 1 in chapter 5 describes two MLC patients who have a premature stop-codon mutation in one or both alleles (p.Tyr71X). Eventhough stop-codon read-through will produce mutant MLC1, this mutant protein might have residual chloride channel activity and, therefore, might still help to alter the disease course. Studies in muscle cell cultures of Duchenne muscular dystrophy patients or mdx mice (mouse model for Duchenne muscular dystrophy) already showed that PTC124 can be useful as therapeutic agent. If PTC124 is able to cross the blood brain barrier, this drug might also be beneficial in MLC patients with nonsense mutations.

Curcumin is the principal curcuminoid of the Indian curry spice turmeric. Curcumin is known for its anti-tumor, antioxidant, anti-amyloid and anti-inflammatory properties. In 2004 Egan et al. reported on the potential use of curcumin as a novel drug for the treatment of Cystic Fibrosis (CF) patients. Curcumin increases the expression of membrane proteins in the plasma membrane. CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) that functions as a chloride channel and controls the regulation of other transport pathways. The most common mutation (p.Phe508del) in CF patients results in the production of a misfolded protein that is retained in the endoplasmic reticulum and targeted for degradation. Egan et al. demonstrated that oral curcumin treatment leads to successful targeting of mutant CFTR to the plasma membrane and partially restoring CFTR function in CFTR mice and baby hamster kidney cells. The data presented in this paper suggest that curcumin might also prove to be useful as a therapy for MLC patients with mutations that cause protein misfolding and reduced membrane expression. Preliminary results of cells treated with curcumin and overexpressing mutant MLC1 (Ser280Leu) show indeed increased membrane expression (personal communication Raul Estevez). Further experiments, however, are needed to test whether increased membrane expression of MLC1 also leads to partial recovery of the function.
10. Future experiments with animal models

To unravel the exact mechanism behind myelin and astrocytic endfeet vacuolation and to test possible therapies, the use of animal models will be indispensable. If the knock-out of MLC1 in the mouse leads to vacuolation of myelin and astrocytic endfeet, this model can be used to study the development of vacuoles during the deposition of myelin by sacrificing the animals at different embryonic and postnatal stages.

An animal model can also be used to further support the existence of a functional relationship between aquaporins and MLC1. In embryonic brain the presence of a large extracellular volume allows the diffusion of water and ions through vessels not surrounded by astrocytes. Later during development and blood-brain barrier differentiation, the glial processes envelop endothelial cells and form a perivascular sheath. When this process comes to an end, the extracellular space is dramatically reduced. To reach a volume fraction typical of adulthood the development of specific transporter mechanisms for water and ions is required. Interestingly, rodents start to buffer extracellular potassium during the first 2-3 weeks of life in correspondence with a marked increase in AQP4 levels.

If there is indeed a functional relationship between MLC1 and AQP4, we can hypothesize that the expression levels of MLC1 are in correspondence with the increased levels of both AQP4 and Kir4.1. This hypothesis can be tested by investigating the expression levels of MLC1, AQP4 and Kir4.1 in mice by sacrificing the animals at different time points during development and especially at the time the blood-brain barrier is formed.

More distant future experiments with a MLC1 knock-out mouse model can be MLC1 gene transfer and cell transplantation. The adeno-associated virus mediated gene transfer to the brain has been used in an ASPA knock-out mouse, which is a model for the leukodystrophy known as Canavan disease. In this knock-out mouse the improvements observed in MRI of the brain were sustained till 3–5 months after injection. A disadvantage of this approach is that the effect of the gene transfer is predominantly present close to the injected site. It is doubtful whether the gene transfer occurs in the entire central nervous system, and if the disease is modified in all areas.

Another approach to recover the lost MLC1 function is cell therapy. This method has been already been used with some success in the ASPA knock-out mouse brain, in which the implanted neural progenitor cells have been shown to be able to differentiate into oligodendrocytes and astrocytes. Implantation of neural progenitor cells is a promising option to recover the lost MLC1 function.
References

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