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論文内容の要旨
Summary of thesis contents

Astrocytes are one of the major glial cells in the CNS and maintain brain milieu through the blood brain barrier formation, uptake of neurotransmitters and supply of nutrients to neurons. In addition to this well-known function, astrocytes may be involved in the white matter development and /or maintenance. This idea came from the analysis of several glial specific gene deficient mice and human diseases. Glial specific genes, connexin and inwardly rectifying potassium channel (Kir4.1), are involved in the homeostasis of potassium concentration,. It has been reported that deletion of connexin or Kir4.1 in glial cells results in the leukodystrophy in mice, (Lutz et al, 2009 ; Mognotti et al, 2011 ; Odermatt et al, 2003 ; Menichella et al, 2006). Moreover, a human disease, Alexander disease, is caused by missense mutations in the GFAP gene, leading to infantile onset leukodystrophy. These previous studies support the idea that astrocyte dysfunction can cause leukodystrophy. However, studies on the relationship between astrocyte and the white matter development/maintenance are still rare. One of the limitations is a lack of mice recapitulating leukodystrophy phenotype through astrocyte dysfunction. In this study, I generated a mouse model for a human disease, Megalencephalic leukoencephalopathy with subcortical cysts (MLC, OMIM604004), to study whether astrocyte dysfunction can lead to leukodystrophy. MLC (OMIM604004) is a rare autosomal recessive neurological disorder with infantile onset characterized by chronic white matter edema, macrocephaly, slowly progressive deterioration in motor function, cerebellar ataxia, spasticity, and mental decline. Two genes, encoding Mlc1 and GlialCAM, were identified to be responsible for MLC (Leegwater et al, 2001; López-Hernández et al, 2011). Mutations in the Mlc1 gene were reported in about two-thirds of MLC patients (Leegwater et al, 2002; van der Knaap et al, 2012). Various mutations in the Mlc1 gene (missense mutations, frame shift and splice site

mutations) have been reported in human MLC patients. However, correlation between types of Mlc1 mutations and the severity of MLC clinical symptoms has not been clarified (Leegwater et al, 2002).

Human Mlc1 and murine Mlc1 genes encode a membranous protein with eight transmembrane domains, whose function is unknown. Northern blot analyses demonstrated that both human Mlc1 and murine Mlc1 genes were predominantly expressed in the brain and in situ hybridization demonstrated that Mlc1 mRNA is present in astrocyte lineage cells including astrocytes, ependymal cells and Bergmann glia, but not in oligodendrocytes (Schmitt et al, 2003; Teijido et al, 2004). Thereby, astrocytic abnormalities should be involved in the leukodystrophy of MLC patients. Studies using the heterologous expression of Mlc1 gene demonstrated that Mlc1 mutants failed to be transported to the plasma membrane (Duarri et al, 2008; Teijido et al, 2004). In addition, biopsy samples from a MLC patient harboring missense mutation displayed low expression levels of Mlc1 protein (Teijido et al, 2004). These data suggested that Mlc1 mutants show a loss-of-function effect, and the model animal could be established by generating Mlc1 null mouse.

To validate this hypothesis, I generated Mlc1 null mouse in which STOP-tetO cassette is knocked-in to the Mlc1 locus. I confirmed that endogenous expression of Mlc1 becomes below the detection level in the STOP-tetO knocked-in homozygote. However, Mlc1 null mouse was viable at least till 18 month of age with normal fertility and showed no behavioral and histological abnormalities. These results indicated that leukodystrophy as seen in human MLC was not recapitulated by the Mlc1 deficiency at least in mice.

More importantly, whether MLC1 mutations lead to loss-of-function or gain-of-function was not well addressed so far. Interestingly, some of glia specific gene-related diseases that are inherited in a Mendelian manner were modeled by wild type gene overexpression in mice. Thereby, I generated Mlc1 over expressing mouse. I took advantages of tetracycline-controlled gene induction system and confirmed Mlc1

overexpression (Mlc1 OE) was achieved in astrocyte lineage cells including astrocytes, ependymal cells and Bergmann glia. Embryonic malformation was not observed in Mlc1 OE mouse even when the Mlc1 overexpression is induced from mid-embryo. At birth the size and the body weight were indistinguishable between Mlc1 OE and the control groups. Mlc1 OE mouse showed growth retardation and mild ataxic gait transiently appeared around postnatal two weeks but these symptoms caught up to the control by three month of age. Moreover, Mlc1 OE mouse was viable at least till 18 month of age, and reproduction was not affected. I then performed histological analysis and found that leukodystrophy is present in the white matter, especially in the corpus callosum and the globus pallidus. Leukodystrophy in the CC appeared from postnatal one to two weeks, and persisted for the whole life. Histological analysis indicated that area with leukodystrophy contained numerous vacuole-like structures by the light microscopic observation. This result indicated that leukodystrophy as seen in human MLC was recapitulated in the mouse when Mlc1 overexpression was induced in astrocytes. By using Mlc1 overexpressing mouse, I studied the process of leukodystrophy formation.

I then examined the ultrastructural feature of the leukodystrophy formation by electron microscopy, and compared them with those of MLC patients. Vacuole-like structure as observed in the light microscopic study was composed of both astrocytic swelling and oligodendrocyte-associating vacuoles. These ultrastructural findings were relevant to those of human MLC. By manipulating the timing of tTA-mediated Mlc1 overexpression from P28 to P38 by DOX administration (OEP28-P38), I found that the astrocytic swelling, but not oligodendrocyte-associated vacuolus, correlated with the extent of tTA-mediated Mlc1 overexpression. Moreover, astrocytic swelling and oligodendrocyte-associated vacuoles were dramatically improved when Mlc1 overexpression was cancelled after completion of the leukodystrophy formation (OEembryo-P28), resulting in the regression of leukodystrophy lesions. Interestingly, Mlc1 overexpression induced only during young

adult stage (OEP28-P90), astrocytic swelling occurred in accordance with the induction of tTA-mediated Mlc1 OE, but niether oligodendrocyte-associated vacuoles nor leukodystrophy phenotype were observed. These results indicated that Mlc1 overexpression primarily caused astrocytic swelling and affected the white matter formation in infantile mouse. However, leukodystrophy lesions did not appear when Mlc1 overexpression was induced after young adult stage even though the astrocytic swelling was present in the white matter, suggesting astrocytes are involved in the white matter development and/or maintenance at the critical period.

Finally, to validate the cell autonomous effect of overexpressed Mlc1, I searched for Mlc1 interacting proteins. Because of the lack of immunoprecipitation compatible anti-Mlc1 antibody, I generated primary antibodies recognizing mouse Mlc1 N-terminus and C-terminus, respectively. By using newly developed anti-Mlc1 antibodies, We screened for Mlc1 interacting proteins by immunoprecipitaton and mass spectrometry analysis, and identified Na+/K+ ATPase α subunits, a member of P-type ATPase, as a Mlc1 interacting protein. Na+/K+ ATPase α subunit is a component of sodium pump, which is essential for generating electrochemical gradients across the cell membrane. Interestingly, pharmacological and genetic studies indicated that mice with reduced sodium pump activity displayed astrocytic swelling relevant to Mlc1 OE mouse. Thus, I examined whether overexpressed Mlc1 affects sodium pump property using cultured astrocytes. This study indicated that sodium pump activity was reduced in Mlc1 overexpressing astrocytes without alterating the amount of Na+/K+ ATPase □ subunits. Reduced sodium pump activity could be reflected by the decreased cell surface expression of sodium pump. Thereby, I measured the amount of cell surface Na+/K+ ATPase □ subunits by 3H-ouabain binding assay. Unexpectedly, the amount of cell surface Na+/K+ ATPase □ subunits increased in the Mlc1 overexpressing astrocyte. Since oigomerization of Na+/K+ ATPase □ subunits and □ subunits on the endoplasmic reticulum is essential for stabilization of the □ subunit, trafficking of the □ subunit to the plasma membrane

and functional maturation as a pump may have been affected in the Mlc1 overexpressed astrocytes. However, Mlc1 overexpression did not alter the amount of Na+/K+ ATPase □ subunits, thereby disturbance of □-□ oligomerization in the endoplasmic reticulum is unlikely to be involved in the reduction of sodium pump activity in the Mlc1 overexpressing astrocyte. Moreover, no evidence for Mlc1 interaction with Na+/K+ ATPase β2 subunit was obtained. From these findings, I propose that 1) overexpressed Mlc1 formed “Mlc1-□ subunit complex”, resulting in the increased amount of the cell surface Na+/K+ ATPase □ subunits, 2) increased “Mlc1-□ complex” formation disturbed “□-□ subunits complex” formation, resulting in lowered Na+/K+ ATPase pump activity. In this study, I generated Mlc1 over expression mouse to examine relationship between the astrocytes and the white matter development, which showed leukodystrophy phenotype relevant to MLC, and found that astrocytes dysfunction is involved in the leukodystrophy formation via impairment of the sodium pump activity. My study will open a new insight into the relationship between astrocytes and the white matter abnormality.

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博士論文の審査結果の要旨

Summary of the results of the doctoral thesis screening

アストロサイトは中枢神経系に存在するグリア細胞の一つであり、ニューロン活動の支持や血液脳関門の形成に寄与することが広く知られている。他方、アストロサイトの異常に起因する疾患では、白質脳症をきたすことが報告されており、脳白質の発達や維持にもアストロサイトが寄与することが示唆されている。しかしながら、白質脳症を発症するモデルマウスの報告は乏しく、アストロサイトと脳白質の発達・維持との関連に着目した研究はなされてこなかった。出願者は、アストロサイトの脳白質の発達・維持への関与を明らかにするために、アストロサイト特異的に発現する MLC1 遺伝子の変異によって白質脳症をきたす Megalencephalic leukoencephalopathy with subcortical cysts(MLC)と呼ばれるヒトの疾患に着目し、そのモデルマウスの作成を試みた。まず、出願者は Mlc1 発現欠損マウスと Mlc1 をアストロサイトに過剰発現させた。（Mlc1 過剰発現マウス） 2 つの遺伝子改変マウスを tet-off システムを改良した方法(Tanaka et al., 2010)で作成、解析した。Mlc1 過剰発現マウスの脳切片において白質ジストロフィーを示すことを見いだし、電子顕微鏡観察により病変の主要素が膨化したアストロサイトと空胞化したミエリンであることを明らかにした。この所見はヒト MLC の所見と類似するものであった。また、ドキシサイクリン投与により Mlc1 過剰発現の期間を操作することで、Mlc1 の過剰発現によって一次的にアストロサイトの膨化が生じ、これに引き続いてミエリンの空胞化および白質ジストロフィーが生じることを示した。この形態学的解析の結果から、出願者は過剰発現された Mlc1 が他の機能分子に対して dominant negative 様に作用しアストロサイトの膨化をきたすという仮説をたてた。この仮説を明らかにするために、生化学的手法を用いて Mlc1 結合蛋白質の同定を試み、sodium pump の構成要素である Na+/k+ ATPase α subunits が Mlc1 結合蛋白質の一つとして同定された。また、sodium pump の活性低下をきたすマウスにおいて、アストロサイトの膨化が生じることが既に報告されており、Mlc1 過剰発現マウスの表現系と類似するものであった。そこで、アストロサイト初代培養細胞を用いて sodium pump 活性の測定を行ったところ、Mlc1 過剰発現マウス由来アストロサイトにおいて sodium pump 活性が有意に低下していた。また、Na+/k+ ATPase α subunits が sodium pump として機能するには Na+/k+ ATPase β subunit と二量体を形成することが重要であることが知られているが、Mlc1 が β subunit と結合するという結果は得られなかった。以上のことから、in vitro において過剰発現 Mlc1 は Na+/k+ ATPase α subunits と結合し、sodium pump の α―β 二量体の形成あるいは維持に対して阻害的に作用し、結果として sodium pump の活性が低下することを示した。

本研究において出願者は、アストロサイトの膨化に起因して白質ジストロフィーをきたすモデルマウスを確立した。また、形態学的、生化学的、細胞生物学的解析から、Mlc1 過剰発現におけるアストロサイトの膨化のメカニズムの一部を明らかにした。これらの結果は、脳白質の発達・維持におけるアストロサイトの重要性を提起するものであった。以上のことから本論文は学位論文に値するとの結論に至った。