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論　文　名	Pathological Studies on the Roles of CCDC85C Protein in Neurogenesis, Gliogenesis, and Ependymogenesis in the Rat Model of Hydrocephalus (水頭症ラットモデルを用いた神経発生, グリア発生および上衣細胞発生における CCDC85C 蛋白の役割に関する病理学的研究)
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論文要旨

Introduction

Coiled coil domain containing 85c (*Ccdc85c*) is a causative gene for genetic hydrocephalus and subcortical heterotopia with frequent brain hemorrhage. To definitively determine the function of *Ccdc85c*, *Ccdc85c* knockout (KO) rat with an F344 genetic background has been established in our laboratory as a new hydrocephalus model. Roles of CCDC85C protein in the histogenesis and pathogenesis for hydrocephalus remain to be elucidated. In this study, the author aimed to analyze a complete understanding of the relations of CCDC85C protein expression with intermediate filament (IF) proteins during lateral ventricle development and the roles of CCDC85C on neurogenesis, gliogenesis, and ependymogenesis in the rat hydrocephalus model in relation to IF protein expression, ventricular zone disruption, and periventricular pathology.

Chapter 1: Expression of CCDC85C and intermediate filament proteins during lateral ventricle development in rats

Section 1: Expression of CCDC85C protein during lateral ventricle development

To analyze the expression pattern of CCDC85C, immunofluorescence was done chronologically in the embryonic and early postnatal rat brains. Using F344 rats, CCDC85C immunofluorescence from fresh frozen brain tissues revealed meshwork-like immunoreactivity in lining the wall of the lateral ventricle during embryonic and early postnatal development. Throughout the embryonic development, CCDC85C expression increased, then rapidly declined after birth and it essentially disappeared at postnatal day 20. The expression of CCDC85C signals was localized at cell-cell junctions of the lateral ventricle wall by immunoelectron microscopy.

Section 2: Expression of intermediate filament proteins during lateral ventricle development

In the dorsal wall of the lateral ventricle, nestin immunostaining was seen in the neural stem/progenitor cells and processes of the radial glia extending from the ventricular zone towards the cortical zone. Nestin expression began to gradually decline in the radial processes and lining cells of the ventricular wall immediately after birth. At postnatal day 20, no nestin expression was seen except in some discrete round cells lining the lateral ventricle. During perinatal development, vimentin immunoreactivity was prominent along the radial processes. A major portion of the ventricular lining cells in the wall of the lateral ventricles was also positive for vimentin. At postnatal day 20, vimentin was localized only in the lining of the lateral ventricle wall. No glial fibrillary acidic protein (GFAP) expression was found in the dorsal wall of the lateral ventricle before birth. Immediately after birth, expression was observed in the astrocytes of the ventricular/subventricular zone (V/SVZ). GFAP expression was upregulated with age and showed a star-shaped pattern. Some circular cells lining the ventricle, such as B1 cells (a subset of V/SVZ astrocytes), were also positive for GFAP. The embryonic and early postnatal lateral ventricles did not exhibit any immunoreactivity when stained with cytokeratin. However, cytokeratin expression started lining the lateral ventricle of the rat brain at around two weeks of age and increased with age.

In comparison to IF protein, immunoreactivity of CCDC85C protein is directly correlated with intensity of nestin and vimentin expression but inversely correlated with GFAP expression during embryonic and early postnatal brain development.

Chapter 2: Disrupted neurogenesis, gliogenesis, and ependymogenesis in the *Ccdc85c* KO rat for hydrocephalus model

Section 1: Disrupted neurogenesis and gliogenesis

To assess the role of CCDC85C on neurogenesis, the author conducted chronological nestin immunohistochemistry during postnatal development. The dorsolateral ventricle wall of KO rats had nearly no stem/progenitor cells and very few disordered radial processes, which denotes radial glial demise. Maldevelopment was detected lining the dorsal region of the lateral ventricle. Furthermore, KO rats began expressing aberrant nestin in the subventricular zone and cerebral cortex at late postnatal ages, indicating ectopic nestin expression, whereas nestin expression almost disappeared in the dorsal lateral ventricle of

control rats at around three weeks. These results showed that nestin expression was altered, and ectopic expression was seen during postnatal periventricular development in KO rats. This suggests that neurogenesis is disrupted in the early postnatal period in KO rats.

Gliogenesis follows neurogenesis during central nervous system development and produces astrocytes and oligodendrocytes. When KO rats were compared to age-matched control rats, GFAP immunohistochemistry revealed a different expression pattern. In the KO rats, there were only a few numbers of GFAP immunolabels lining the ventricle at early postnatal development, with essentially no cortical immunostaining. GFAP expression in the KO rats became more prominent with advancing age. Astrogliosis with increased length and branching of astrocytic processes was noted. All ages of KO rats showed maldevelopment of ventricular lining cells. Oligodendrocyte transcription factor 2 (Olig2), a marker for oligodendrocytes, was more prevalent in KO rats at early stage after birth. At late stage, however, expression matched that of controls. Periventricular neuron glia antigen-2 (NG2)-positive cells, an oligodendrocyte precursor cell, were increased in the KO rats. An increased number of Iba1, a marker for microglia and macrophages, was observed in the periventricular area of the dorsal lateral ventricle, whereas activation of microglia and macrophages was absent in the control. These results indicate abnormalities of GFAP expression as well as astrogliosis, disturbed oligodendrogliosis, and increased microglia in the developing lateral ventricle of the KO rats. Therefore, CCDC85C plays a critical role in gliogenesis.

Section 2: Disrupted ependymogenesis

On vimentin immunohistochemical investigation, KO rats had weaker vimentin immunoreactivity compared to age-matched control rats. The maldevelopment of the ventricular lining ependymal cells was evident at all ages. Around four weeks of age, there was some ectopic vimentin expression in the subventricular zone. These ectopic expressions were never seen in the control rats. The ventricular lining cells of the KO rats lacked expression of cytokeratin at any age. Ectopic cytokeratin expression was found in the area surrounding the dorsolateral ventricle around four weeks of age. When the ependymal cilia and ventricular junction were examined using α-tubulin (a marker for cilia), ciliary rootlet coiled-coil (CROCC-a root marker), and N-cadherin (a neuronal cell adhesion marker), the author found loss of cilia, roots, and loss of expression of neuronal adhesion markers with denudation of the ependymal lining in the wall of the dorsal lateral ventricle. α-tubulin immunohistochemistry strongly supported the loss of ependymal cilia. These results suggest that CCDC85C has a significant role in ependymogenesis.

Chapter 3: Periventricular abnormalities during lateral ventricles development in the *Ccdc85c* KO rats

Section 1: Aberrant expression of α-smooth muscle actin in periventricular area of the lateral ventricle

The author observed aberrant expression of α-smooth muscle actin (α-SMA) in the periventricular area of the KO rats. Following that, the author conducted serial-section immunohistochemistry using nestin, vimentin, GFAP, and cytokeratins. When compared to the expression of α-SMA, each of these markers showed a distinct pattern. Aberrant expression of α-SMA started around three weeks of age, when hydrocephalus is prominent in KO rats. Then the origin of α-SMA expressing cells was analyzed using double immunofluorescence with nestin, vimentin, GFAP, cytokeratin, desmin, and NG2 (a pericyte marker). Aberrant α-SMA was co-expressed with nestin, vimentin, and GFAP with a few NG2, but not co-expressed with cytokeratin and desmin. These findings indicate immature glial cells might be present around the lining of the lateral ventricles of the KO rats.

Section 2: Periventricular vascular pathology

Abnormal expression of von Willebrand factor (a marker for vascular endothelium) was seen in the periventricular blood vessels of the lateral ventricle. A split basement membrane was seen in the vessels of the periventricular area of the dorso-lateral ventricles when laminin and collagen IV were immunolabeled. FITC-labeled tomato lectin injection through the abdominal vein also showed abnormal vasculature on the wall of the dorsal lateral ventricle.

Chapter 4: Comprehensive discussion for the pathogenesis of neurogenesis, gliogenesis, and ependymogenesis in *Ccdc85c* KO rat, a model of hydrocephalus

Genetic studies in animal models have started to open the way for understanding the underlining pathology of hydrocephalus. The Hydrocephalic Texas Strain (HTX) rat model is characterized by ventricular enlargement and closure of the aqueduct, which subsequently develop hydrocephalus. Hydrocephalus with hop gait (*hyh*) mouse mutation results in dilated lateral ventricles and a large third ventricular cyst with obstruction of the cerebral aqueduct. *Ccdc85c* knockout rats show subcortical derangement with communicating aqueduct. In present study, the author discovered, *Ccdc85c* knockout rat has the ventricle system with embryonic characteristics at its developing stages around three weeks of age when hydrocephalus is prominent.

Conclusions

Based on these results, the following conclusions are obtained:

1. Immunoreactivity of CCDC85C protein expression is directly proportional to intensity of nestin and vimentin expression but inversely proportional to GFAP expression during the embryonic and early postnatal development of the brain (Chapter 1).
2. Altered and ectopic expression of nestin, vimentin, and cytokeratin in the wall of the dorsolateral ventricle in the KO rats with maldevelopment of the ependymal cells and loss of cilia (Chapter 2).
3. Disturbed GFAP, Olig2, and NG2 expression with increased reactive microglia/macrophage surrounding the periventricular area of the developing KO rat brain (Chapter 2).

4. KO rats show disrupted ventricular formation and subventricular zone cell junctions (Chapter 2).
5. Aberrant expression of the mesenchymal marker α-SMA started around 3 weeks of age in the periventricular area of the lateral ventricle of the KO rats, when hydrocephalus is prominent (Chapter 3).
6. Immature glial cells might be present in the periventricular area of the lateral ventricle of the KO rats with damaged and split basement membraned vessels (Chapter 3).
7. To the best of author's knowledge, aberrant α-SMA expression was not reported previously in hydrocephalus or other neurodevelopmental diseases (Chapter 3).
8. Taken together, CCDC85C is expressed in the cell-cell junctions lining the wall of the lateral ventricle and plays important roles in neurogenesis, gliogenesis, and ependymogenesis with intermediate filaments and other related proteins during the embryonic and postnatal development of the brain.

審査結果の要旨

水頭症は脳脊髄液が脳室内に貯留して脳内の圧力が高まり、脳室の拡張に伴う脳の機能や発達障害を引き起こす疾患である。ヒトの先天性水頭症は出生異常の中でも発症頻度が高く、新生児の約 1000 人に 1 人の割合でみられる。水頭症の病理発生の解明には、モデル動物が貢献すると期待されている。

Hemorrhagic hydrocephalus (*hhy*) マウスは、大阪府立大学理学部の動物施設にて発見された出血を伴う先天性水頭症を示すミュータントマウスであり、Coiled-coil domain-containing 85c (*Ccdc85c*) が原因遺伝子である。*Ccdc85c* 遺伝子の機能を解析するためには、複数の動物種を用いて多面的な機能解析が必要である。Transcriptional activator like effector nuclease (TALEN) を用いたゲノム編集によって F344 系統を背景系統とする *Ccdc85c* ノックアウト（KO）ラットが作製された。本研究では、CCDC85C 蛋白発現と細胞マーカーとなる中間径フィラメント蛋白の発現を詳細に調べ、*Ccdc85c* KO ラットの神経発生、グリア発生、上衣細胞形成における *Ccdc85c* 遺伝子の役割を解析した。

第 1 章では、野生型 F344 ラットを用いて CCDC85C 蛋白発現と中間径フィラメント発現を解析した。胎生期には CCDC85C 蛋白は脳室壁を覆う網目構造としてみられ、陽性所見は細胞間に局在していた。CCDC85C の発現は生後に急速に減弱し、生後 20 日目には消失した。中間径フィラメント蛋白であるネスチン、ビメンチン、GFAP およびサイトケラチンと CCDC85C 蛋白の発現推移を比較検討した。ネスチンおよびビメンチンは未分化神経細胞である放射状グリアの突起にみられ、脳の発達とともに減弱した。一方、アストロサイトマーカーの GFAP は脳発達につれて出生後に発現が強くなり、成熟アストロサイトの形態を示すようになった。サイトケラチン陽性細胞は生後約 2 週齢で側脳室の内側を覆い初め、脳の発

達とともに発現が増強した。CCDC85C 蛋白質の免疫反応性は、ネスチンおよびビメンチン発現強度と相関していたが、GFAP 発現とは逆相関していた。

第 2 章では、*Ccdc85c*KO ラットにおける神経発生、グリア発生および上衣細胞形成を解析する目的で、CCDC85C 蛋白と中間径フィラメント発現を調べた。KO ラットの異所性灰白質が形成される背側の脳室壁には神経幹細胞がほとんどなく、少数の放射状グリアのみがみられた。KO ラットの生後初期における GFAP 発現細胞は少数で、Olig2 陽性のオリゴデンドロサイトは増数していたが、脳の発達とともにアストログリオーシスへと進展した。水頭症の病変の進行ともに、脳室周囲の Iba1 陽性のミクログリア／マクロファージの増数がみられた。

KO ラットでは、脳室を覆う上衣細胞の発達不全がみられ、線毛マーカーα-tubulin、ルートレットマーカーCROCC、細胞接着マーカーの N-cadherin の発現を欠いており、線毛の消失と上衣細胞の発達異常・脱落が示された。KO ラットの脳室周囲にはサイトケラチンやネスチン陽性細胞の異所性発現が認められた。これらの所見より、KO ラットでは生後初期に脳室周囲の神経発生が障害されることが明らかとなり、CCDC85C 蛋白発現が神経発生、グリア発生、上衣細胞形成に重要であることが示された。

第 3 章では、*Ccdc85c*KO ラットの脳室周囲領域で生後 3 週齢頃からα-SMA の異常な発現がみられたため、この異常細胞の特徴を調べた。異常なα-SMA 陽性細胞は、ネスチン、ビメンチン、GFAP および少数の NG2（血管周囲細胞マーカー）と共発現したが、サイトケラチンおよびデスミンとは共発現しなかった。これらの所見は、*Ccdc85c*KO ラットの側脳室周囲に未熟なグリア細胞が存在する可能性を示した。

さらに、*Ccdc85c*KO ラットにおける脳出血の病理発生を明らかにするために、脳室周囲血管の血管病変を von Willebrand factor (vWF：血管内皮マーカー)、ラミニンおよびコラーゲン IV(どちらも基底膜のマーカー)を用いた免疫組織化学および FITC 標識トマトレクチン（血管内皮マーカー）を用いて解析した。側脳室の脳室周囲の血管周囲に異常な vWF の陽性所見がみられ、背外側の脳室周囲の血管では、ラミニンおよびコラーゲンIV陽性の離断して二重となった基底膜がしばしば観察された。FITC 標識トマトレクチンを後大静脈から注射後に脳組織をサンプリングして蛍光観察したところ、背側側脳室周囲の血管において血液の漏出を示す異常を認めた。

第 4 章では、これまで水頭症モデル動物として報告されている Hydrocephalic Texas Strain (HTX)ラットおよびホップ歩行を伴う水頭症マウス（*hyh*)との病態比較を含めた総合考察を行っている。*Ccdc85*KO ラットは、これまでに報告されていない脳室系の発達異常と異所性灰白質形成を特徴としており、新たな水頭症モデルとして有用であることが示された。

本研究は新規の水頭症モデルマウスから同定された水頭症原因遺伝子 *Ccdc85c* の生物学的機能を明らかにする目的で、*Ccdc85c*KO ラットにおける水頭症発達の病態を中間径フィラメント発現に注目して解析している。今回得られた成果は、*Ccdc85c* 遺伝子の神経発生、グリア発生および上衣発生における生物学的機能の一端を明らかにしており、基礎獣医学ならびに基礎医学の発展・展開に貢献するものと考えられる。従って、本論文の審査ならびに最終試験の結果と併せて、博士（獣医学）の学位を授与することを適当と認める。