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論文内容の要旨
Summary of thesis contents

Role of Cathepsin C and Cystatin F in demyelinating diseases

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Introduction

There are many types of diseases that damage myelin in the central nervous system (CNS). Multiple sclerosis (MS) is the most common demyelinating disease in the CNS. In this disorder, the immune system attacks the myelin sheath and causes inflammation and injury to the sheath and ultimately to the nerve fibers that it surrounds. The results are multiple areas of scarring (sclerosis), where premyelinating oligodendrocytes fail to remyelinate axon at affected area. Studies on therapeutic attack on MS have been significantly increased in number in recent years and improved therapeutic outcome. Animal models have been critically important for addressing and establishing MS treatment. There are several animal models that can be used for the study. In this study, the heterozygous transgenic 4e ($plp^{4e/-}$) mouse and the experimental allergic encephalomyelitis (EAE) model were selected to study different demyelination phases.

The chronic phase study used the $plp^{4e/-}$ *mouse*, in which the proteolipid protein gene is overexpressed. This mouse model starts demyelination accompanied by myelin regeneration from 2 months old. The regeneration of myelin will stop at 6 months old but demyelination still proceeds. At an early phase, $Plp^{4e/-}$ mouse model shows pathology with no immune response involvement, therefore the inductive event is different from that of MS. However, at later phase, they have similar pattern of pathological events, in which premyelinating oligodendrocytes fail to remyelinate axons. This is similar to the observation in chronic phase of MS. We previously found that Cathepsin C (CatC) is upregulated in microglia of demyelination models, especially in chronic demyelinated lesions in $Plp^{4e/-}$ mouse. Additionally, its inhibitor Cystatin F (CysF), which is a cysteine protease inhibitor and is also induced during early phase of demyelination, ceased expression in chronic demyelinated lesions. The myelin oligodendrocyte glycoprotein (MOG) induced EAE is most commonly used as a laboratory model for MS. Inductive phase of EAE shows pathology and progression of disease similar to those observed in MS, but in the final phase mouse gain full recovery. Thus, I selected EAE mouse to study acute demyelination phase. The study on the role of CatC and CysF played in demyelinating diseases should be useful for development of effective new therapeutic target of MS or other demyelinating diseases.

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Materials and methods

To study the role of CatC and CysF in demyelinating disease, we generated mouse line to manipulate CatC and CysF expression by using Flexible Accelerated STOP Tetracycline Operator Knockin (FAST) system (Tanaka et al, 2010). The homozygous knock-in mouse (*CatC*$^{STOP/STOP}$, *CysF*$^{STOP/STOP}$) showed no expression of CatC or CysF, which can be considered as CatC or CysF knockdown (CatCKD or CysFKD) mice. CatCKD and CysFKD transgenic mice were crossed with *Iba*$^{tTA/-}$ and *Plp*$^{4e/-}$ mouse to overexpress CatC or CysF in microglia (CatCOE and CysFOE) of *Plp*$^{4e/-}$ mouse for the study in the chronic phase. For acute phase, MOG-EAE model was used in the wild type, CatCKD and CatCOE mice. The MOG35–55 peptide (MVGWYRSPFSRVVHLYRNGK, 150 μg) emulsified in complete Freund's adjuvant, consisting of incomplete Freund's adjuvant with 4 mg/ml Mycobacterium tuberculosis. Mice were immunized subcutaneously with MOG/CFA into pelvic region followed by injection intraperitoneally with 400 ng pertussis toxin immediately (Day 0) and 48 hours later (Day 2). The severity of EAE was daily monitored and graded as the clinical score.

Result

To study the role of CatC and CysF in demyelinating diseases, we generated transgenic mouse lines in which CatC or CysF expression can be manipulated by the FAST system in this study. CatCKD, CysFKD, CatCOE and CysFOE mice were observed for any defects that arrise from gene manipulation. None of the transgenic mice showed any physical phenotype or clinical symptoms but showed activated microglia phenotype. From previous studies, CysF is upregulated in ongoing remyelinating phase but CatC conversely tends to dominate CysF in the chronic phase of PLP transgenic mouse. CysF plays a main role during early remyelinating phase. In ongoing remyelination phase of *Plp*$^{4e/-}$ mouse at age 4 months, CysFKD mouse significantly enhanced demyelination, while no effect were found in CatC deprived *Plp*$^{4e/-}$ mouse at the same time point of an early phase. I confirm this result by changing the balance of CatC and CysF expression by overexpressing CatC in microglia of *Plp*$^{4e/-}$ mouse. I found that CatCOE mouse also resulted in a similar phenotype with CysFKD mouse showing early demyelination appearance. Conversely, during the chronic demyelination phase at age 8 months, CatCKD mouse diminished demyelination in the *Plp*$^{4e/-}$ mouse. CysF was expressed in the activated microglia, M1 (pro-inflammatory) microglia, but not in M2 (anti-inflammatory) microglia.

To further study towards clinical application, EAE was chosen as an experimental model for the early phase of inflammatory demyelination. I found CatC mRNA-expressing cells in the grey horn and anterior median fissure of spinal cord at an early phase of MOG-EAE models. These CatC mRNA expressing cells were identified as microglia and neutrophils, respectively. From this result, I concluded that CatC should have an important role in pathogenesis of EAE because of their earlier expression at target sites than other molecules. In the late phase of MOG-EAE, CatC was expressed at chronic demyelinating regions but not CysF. CatCKD expression showed less demyelination in the EAE mouse. Conversely, CatC overexpression in microglia significantly enhanced its demyelination. M1 cells were found to accumulate at the demyelination area, which correlated with CatC expression. The result in

MOG-EAE model was similar to the finding in the chronic demyelination model, *PLP*$^{4e/-}$ mouse. Therefore balance of CatC and CysF expression plays an important role in demyelinating diseases in both acute and chronic phases.

Discussion

In the chronic lesion of MS, remyelination process is inadequate, which results in demyelinated lesion. This study demonstrated CysF and CatC are possible crucial factors and the main trigger to inhibit myelin regeneration. During the late phase of chronic demyelinating disease, CatC was found to dominate CysF and full activation is expected. CysF indicates the occurrence of ongoing remyelination (Ma et al., 2011). This might cause impaired myelin regeneration and appearance of naked axons in the *Plp*$^{4e/-}$ mouse (Kagawa et al. 1994). I found CatC knockdown result in more intact myelin in the late phase of chronic demyelination. This gives strong evidence that CatC is one of the main players to induce demyelination. The conclusion of this study determined that balance of CatC and CysF expression plays an important role in chronic demyelinating disease. Several studies revealed important role of microglia/macrophage activation into different cell types, referred to as microglia/macrophage polarization, that results in cells with either pro-inflammatory (M1 cells) or anti-inflammatory properties (M2 cells) (Samuel and Antje 2011). In the late phase *Plp*$^{4e/-}$ mouse, microglia tends to polarize to pro-inflammatory M1 cells. CysF seemed to relate with M1 polarization cells.

I also found CatC-CysF maintain expression along the progression of demyelination in MOG-EAE. CatC was expressed at the chronic demyelination area, where myelin regeneration process is terminated, but CysF was expressed at the edge of demyelination area, where myelin still remains intact. CysF as endogenous inhibitor of CatC attenuate the activation of a wide range of downstream serine proteases involved in inflammation and immunity (Hamilton et al., 2008). CatC and CysF seem to be strongly related to the inflammatory demyelination. This conclusion was confirmed in MOG-EAE-induced CatC knockdown and CatC overexpression mice in this study. The absence of CatC result in easing the severity of EAE, conversely the increment of CatC in microglia enhanced severity in MOG-EAE. These strongly suggest CatC-CysF interaction have an important role in both pathogenesis of inflammatory demyelination in EAE. EAE model is most commonly used as a model in research for intervention therapeutic for MS or other demyelination diseases. The discovery of CatC-CysF system strongly related to inflammatory demyelination might be the key molecules for further development of the new treatment in the future.

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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

髄鞘は軸索を取り囲む密な膜構造であり、中枢神経系ではオリゴデンドロサイトにより形成される。髄鞘は軸索を伝わる伝導速度を著しく速め、脳の高速演算を可能としている。脱髄疾患は髄鞘が変性する疾患であり、中枢神経系では多発性硬化症がその代表的な疾患である。多発性硬化症は自己免疫性疾患であり、髄鞘の構成蛋白質に対する自己免疫反応により髄鞘が攻撃を受け変性するが、初期には髄鞘再生がおきるため、神経症状としては表れにくい。しかし、慢性期に入ると髄鞘再生が阻害され髄鞘のない裸の軸索が多く出現して、種々の神経症状が表れるようになる。カテプシンＣは酵素前駆体のＮ末端２アミノ酸を切断し、これにより多くのセリンプロテアーゼを活性化する。また、出願者が所属する研究室の先行研究から、カテプシンＣ活性を阻害するシスタチンＦの発現が、慢性脱髄巣において著しく減少することを明らかにした。出願者は、この髄鞘再生阻害機構におけるカテプシンＣと阻害因子シスタチンＦの調節作用に着目し、シスタチンＦ遺伝子発現とカテプシンＣ遺伝子発現を各々、完全に抑制、あるいはミクログリアに過剰発現できる遺伝子改変マウスを用いて以下の研究を行った。

出願者はまず、慢性脱髄巣のモデルマウスとしてミエリンプロテオリピド蛋白質過剰発現マウス（PLPtg）を用い、本マウスにおいてシスタチンＦとカテプシンＣが共にミクログリアに発現すること、また髄鞘再生期にはシスタチンＦがカテプシンＣより優性であるが、慢性脱髄期になるとその発現レベルが逆転することを見出した。そこで、シスタチンＦとカテプシンＣの発現バランスを乱す遺伝子操作を行い、脱髄に及ぼす影響を調べた。まず、シスタチンＦの発現を抑制、もしくはミクログリアにおいてカテプシンＣを過剰発現させたところ、髄鞘再生期において髄鞘再生が抑制された。次に、カテプシンＣの発現を抑制したところ、慢性脱髄期において髄鞘再生が継続することを見出した。この結果は、ミクログリアにおけるシスタチンＦとカテプシンＣ発現のバランスが、慢性脱髄巣での髄鞘再生に関与することを示している。

さらに出願者は、実験性アレルギー脳脊髄炎（EAE）マウスを用いて研究を行った。PLPtgは、脱髄に自己免疫反応が関与しないため、脱髄初期の解析には不向きであるが、EAEマウスは多発性硬化症と同じ機序で発症すると考えられ、脱髄初期の解析に用いられる。出願者はまず、EAEマウスにおいてカテプシンＣがシスタチンＦより早期に発現すること、またその発現は灰白質内のミクログリアと、循環系から浸潤した好中球にあることを見出した。次いでカテプシンＣの発現を抑制したところ、EAEマウスの症状や脱髄面積が有意に軽減した。さらに、ミクログリアにカテプシンＣを過剰発現させたところ症状が劇的に悪化した。

以上の結果は、EAEマウスにおいてもカテプシンＣ発現を抑制すると脱髄が軽減することを示唆する。また本研究成果は、多発性硬化症の初期においても、また慢性期においても、カテプシンＣ－シスタチンＦバランスを変化させることが多発性硬化症の新しい治療法の開発につながる可能性を示している。

このように、本研究結果は、2種類の脱髄モデルマウスにおいて、ミクログリアあるいは好中球のカテプシンＣ－シスタチンＦバランスが脱髄の進展に作用を及ぼすことを明らかにした。これらの実験結果は明瞭であり、その科学的価値は大きい。以上の理由から、審査委員会は全員一致で本論文が学位論文として相応しいと判断した。