学 位 論 文 の 要 旨

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論 文 内 容 の 要 旨

INTRODUCTION

The cytosolic carboxypeptidase (CCP) 1, also known as Nna1 (nervous system nuclear protein induced by axotomy protein 1), or *AGTPBP1* (ATP/GTP binding protein 1), mainly encodes an enzyme that deglutamates target proteins, which consist of 789 amino acid residues. A mutation of *CCP1* leads to the degeneration of cerebellar Purkinje and granule cells, which leads to the appearance of a series of pathological signs, such as cerebellar ataxia and cognitive retardation.

Several *Agtpbp1* alleles have been reported in mice, including 8 spontaneous gene mutation mouse models (MGI). The AMS (Ataxia and Male Sterility) mouse has an *ams* point mutation. The AMS mouse is named for its two main clinical manifestations, signs of cerebellar ataxia due to Purkinje cell (PC) degeneration that begin at postnatal day (P) 21 and oligospermia due to defective spermatic differentiation. The maintenance and establishment of the AMS mouse strain can be used to analyze the occurrence and development of the disease in mice before the clinical onset.

PCs are the only neurons that send output signals from the cerebellar cortex. The same progressive degeneration of PCs was also demonstrated in Purkinje Cell Degeneration (PCD) mice, which have the same allele as AMS mice. Studies have shown that β-tubulin polyglutamate is caused by mutations in the *CCP1* gene. Polyglutamylation is a reversible post-translational modification that sequentially adds glutamic acid residues to its target protein to form side chains. Studies have shown that CCP1 can dynamically regulate the balance of polyglutamylation by hydrolyzing the a-carboxyl group of glutamic acid. The imbalance of polyglutamylation results in both structural abnormalities and microtubule dysfunction, which eventually leads to neurodegeneration. The Nna1 knockout (KO) mouse used in this report is a

PCD mouse model in which exons 21 and 22 on chromosome 13 are artificially knocked out. Sheikh et al. have found an increase in the post-translational modification of polyglutamate in AMS mice, which is presumed to underlie PC neurodegeneration. However, the detailed disease progression of neuronal damage caused by CCP1 dysfunction has not yet been elucidated.

In this report, we further monitor the impact of CCP1 dysfunction on the development of different cells in the cerebellum during the pre-onset and clinical stages by examining the wild-type (WT), AMS, and Nna1 KO mice. This study provides supportive data for pre-onset screening and gene targeted therapy for cerebellar development related diseases.

MATERIALS AND METHODS

The AMS mice and Nna1 KO mice were used as disease models, which were bred and maintained in the Institute of Experimental Animals, Shimane University. Mice were deeply anesthetized with pentobarbital (10 mL/kg intraperitoneal injection) and euthanized with a lethal dose of anesthetic agent. Subsequent the mice were collected tissue, fixed, embedded and sliced. Sections (thickness: 4 μm) were stained by hematoxylin and eosin (HE), immunohistochemistry and immunofluorescence for a histopathological examination. Meanwhile, mouse cerebellum specimens were dissected and lysed in RIPA buffer. The extracted protein was detected by WB. As well as we could observe the ultrastructural alterations in tissue specimens by TEM. All experiments with animals in this study were approved by the Animal Care and Use Committee of Shimane University (approval numbers: IZ2-32, IZ2-87, IZ2-88).

RESULTS AND DISCUSSION

In this report, we show that the loss-of-function of CCP1 caused by the mutation of CCP1 triggers a series of neurodegenerative changes in the AMS mouse cerebellum. The AMS mouse can be used to reveal the molecular mechanisms of human CCP1 deficiency to analyze the pathological change of different types of neuronal cells, such as PCs and granular cells in cerebellum, as well as extracerebellar cells (e.g., retinal photoreceptor cells), olfactory mitral cells and spermatogenic cells of the testis. We demonstrated that the lack of CCP1 most likely affects the normal structure and function by perturbing the process of posttranslational modification of microtubules, contributing to morphological alteration of the PCs and progressive cerebellar breakdown.

The histopathology of HE-stained cerebellar tissue of WT mice was examined by light microscopy on postnatal days (P) 7, P15, P21 and P28. The immunohistochemical expression of CCP1 in the molecular and the granular layers of the WT mice was the strongest at P7, before structural completion of the Purkinje cell layer, and decreased at P15. The CCP1 expression was relatively stable after P21. Additionally, positivity in the soma and dendrite of the PC was clearly shown at P15, when the PC layer is discernible, through P28. In comparison to WT mice, the PCs in the cere-bellum of AMS and Nna1 KO mice started to degenerate from P21, after which

the number of PCs decreased and almost completely disappeared by P28. After the statistical analysis of the CCP1 staining intensity in PCs, we found that staining intensity in WT PCs was significantly different from that in AMS and Nna1 KO PCs before P15. However, after the PCs began to degenerate, there was also a statistically significant difference in the intensity of CCP1 staining between the AMS mice and the Nna1 KO mice at P21 and P28 ($p < 0.01$).

IF staining in WT mice highlighted the expression of CCP1, first in the granular layer, and subsequently in the soma and dendrite of PCs at P15 after the structural completion of the Purkinje cell layer. The remaining PCs of AMS homozygous mice after the symptomatic stage (P21 and P28) exhibited a CCP1 signal. Meanwhile, the CCP1 expression in the granular layer of WT, AMS and Nna1 KO mice was stable from P7 to P28, even though they were in the post symptomatic stage.

We next explored the expression levels of CCP1 protein in the cerebellum of WT and AMS mice at postnatal stages (P7, P15, P21 and P28). The Western blotting showed that the level of CCP1 protein in the cerebellum of AMS mice was decreased in comparison to WT mice at each of the four different ages. In the Nna1 KO cerebellum, the CCP1 protein was under a detectable level in this condition. As the band expression of AMS mice is weak, we next increased the sample size and protein quality to analyze the changes of CCP1 protein levels in AMS mice at different ages. Western blotting showed that the expression level of CCP1 in AMS mice was significantly different from that of the WT from P15.

To further confirm the effect of CCP1 gene alteration on the development of PCs, we used transmission electron microscopy to observe the PCs in the mouse cerebellum at four different ages. Electron microscopy revealed that the nuclear membranes of PCs in AMS and Nna1 KO mice showed slight shrinkage and a concave morphology at P15. In comparison to WT mice, the mitochondria and microtubules appeared to have normal structures at this age. At P21 in AMS mice, the following features were observed: the electron density of PC dendrites was increased; the mitochondria were swollen, and their cristae structure was destroyed; the endoplasmic reticulum was expanded to form a vesicle-like structure; the microtubule structure was depolymerized and broken; In addition to the above morphological changes, polysome depolymerization and some lysosomes were also observed in Nna1 KO mouse cerebellar PCs on P21.

CONCLUSION

We provide evidence that point mutations in AMS mice impair CCP1 protein expression and that the expressed protein loses its function. Simultaneous lack of CCP1 most likely affects normal structure and function by disrupting microtubule post-translational modification processes, leading to morphological changes in PCs and progressive cerebellar failure.