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Increased apoptosis and hypomyelination in cerebral white matter of macular mutant mouse brain

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ABSTRACT

Hypomyelination in developing brain is often accompanied by congenital metabolic disorders. Menkes kinky hair disease is an X-linked neurodegenerative disease of impaired copper transport, resulting from a mutation of the Menkes disease gene, a transmembrane copper-transporting p-type ATPase gene (*ATP7A*). In a macular mutant mouse model, the murine ortholog of Menkes gene (mottled gene) is mutated, and widespread neurodegeneration and subsequent death are observed. Although some biochemical analysis of myelin protein in macular mouse has been reported, detailed histological study of myelination in this mouse model is currently lacking. Since myelin abnormality is one of the neuropathologic findings of human Menkes disease, in this study early myelination in macular mouse brain was evaluated by immunohistochemistry. Two-week-old macular mice and normal littermates were perfused with 4% paraformaldehyde. Immunohistochemical staining of paraffin embedded and vibratome sections was performed using antibodies against either CNPase, cleaved caspase-3 or O4 (marker of immature oligodendrocytes). This staining showed that cerebral myelination in macular mouse was generally hypoplastic and that hypomyelination was remarkable in internal capsule, corpus callosum, and cingulate cortex. In addition, an increased number of cleaved caspase-3 positive cells were observed in corpus callosum and internal capsule. Copper deficiency induced by low copper diet has been reported to induce oligodendrocyte dysfunction and leads to hypomyelination in this mouse model. Taken together, hypomyelination observed in this study in a mouse model of Menkes disease is assumed to be induced by increased apoptosis of immature oligodendrocytes in developing cerebrum, through deficient intracellular copper metabolism.

1. Introduction

Hypomyelination is accompanied by various congenital metabolic disorders [1,2] and thought to be related with mental retardation. Proper myelinogenesis is an important step in brain development for intact CNS functions. However, the precise mechanisms of hypomyelination during brain development remain to be elucidated.

Menkes disease (MIM 309400) is an X-linked recessively inherited disorder characterized by progressing neuronal degeneration with copper deficiency due to a defect of a transmembrane copper-transporting p-type ATPase gene (*ATP7A*). Severe hypomyelination is also reported in CNS of Menkes disease patients [3–5]. We employed macular mutant mice, a moderate model of Menkes disease in which the murine ortholog of Menkes gene (mottled gene, *Atp7a*) is mutated [6,7], to gain a better understanding about myelination in neurodegenerative brains. Deficient copper level of macular mouse brain was reported previously [8]. Neurodegeneration pathogenesis in Menkes disease is considered to be related with neuronal apoptosis through disturbance of copper mediated enzymes [9]. In previous studies, neuronal apoptosis has been reported in the brains of Menkes disease mouse models, such as the macular mutant or brindled mouse [10,11]. Although neuronal degeneration is a high concern in this congenital metabolic disease, myelination is another important facet of this disease with its role in normal mental development.

Copper deficiency is known as a causative factor of hypomyelination. For example, in Swayback disease of lambs, degenerative changes in white matter and decreased CNS copper content have been found [12]. Experimental copper deficiency has also been studied using a nutritionally copper deficient rat model in which hypomyelination has been observed with biochemistry and histology [13,14]. In addition, mice treated with oxaldihydrazone (cuprizone), a copper chelating substrate, have degeneration of myelin forming cells as well as their precursor cells and show subsequent demyelination in corpus callosum [15,16].

Since hypomyelination has been reported histologically in optic nerves [17] and biochemically as decreased expression of myelin protein [8,18] in macular mutant mouse, we have hypothesized that degeneration of myelin forming cells or their precursors may play an

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important role in disrupted myelination in this mouse model. Thus, we evaluated cleaved caspase-3 positive cells in white matter, which is assumed to contain degenerating myelin forming cells. Histological examination including immunohistochemistry of myelin protein also employed in this study for estimating detailed myelination in developing macular mouse cerebrum.

2. Materials and methods

2.1. Animals and tissue preparation

Heterozygous macular female mice (C3H-$Mo^{Ml/+}$) were mated with control male mice (C3H-$Mo^{+/y}$). Six hemizygotes (Ml/y) and six male littermates (+/y) were used for this study. The hemizygotes display gradual weight loss and die around postnatal day (PND) 15. At PND 14, mice were anesthetized and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer (fixative buffer) for both paraffin embedded and vibratome sectioning. Brains were removed and post-fixed overnight in the same fixative. Some specimens were processed for paraffin embedding and subsequent sectioning of 5 μm thick coronal sections. Other specimens had 50 μm thick vibratome sections prepared.

2.2. Histology and immunohistochemistry

Vibratome sections were used for immunohistochemistry for cleaved caspase-3 and O4. Sections were pretreated with 3% H_2O_2 in phosphate-buffered saline (PBS) for 15 min at room temperature followed by a PBS wash. Then, sections were incubated with 5% goat or horse serum in PBS at room temperature for 30 min, and were incubated with primary antibodies, polyclonal anti-cleaved caspase-3 antibody (1:1000, Cell Signaling Technologies, Beverly, MA) or monoclonal anti-O4 antibody (1:200, Chemicon, Temecula, CA), overnight at 4 °C. Biotinylated secondary antibodies (1:500, Vector Laboratory, Burlingame, CA) were applied for 2 h and sections were serially stained with avidin–biotin using ABC elite kit (Vector Laboratory, Burlingame, CA). Reaction products were visualized using DAB substrate kit for peroxidase (Vector Laboratory, Burlingame, CA).

Immunohistochemistry for 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) (1:500, Sigma, St. Louis, MO) and glial fibrillary acidic protein (GFAP) (1:1000, DAKO, Glostrup, Denmark) was performed on paraffin sections. After deparaffinization, rehydration, and H_2O_2 blocking, sections were microwaved in 10 mM citrate buffer for 5 min and were stained using the avidin–biotin complex method as described above.

2.3. Statistical analysis

Quantitative estimation of cleaved caspase-3 positive cells in subcortical white matter, internal capsule, and cerebral cortex was performed by counting the number of positive cells in the cerebral hemisphere at the level of the optic chiasm. Two sections per mouse were used (3 mice each for macular and littermate controls).

Student's t-test was used for statistical evaluation.

3. Results

3.1. Myelination in macular mutant mouse brain

Using CNPase immunostaining, relative hypomyelination was observed in white matter of macular mouse, especially in corpus callosum and internal capsule (Fig. 1 A–D). CNPase antibody reacts with the mature myelin sheath and oligodendrocytes. The myelinated area in the cerebral hemisphere of macular mouse was restricted, and fewer myelin sheaths were observed in macular than in control cerebrum. Although myelination is still occurring and a significant area of cerebral

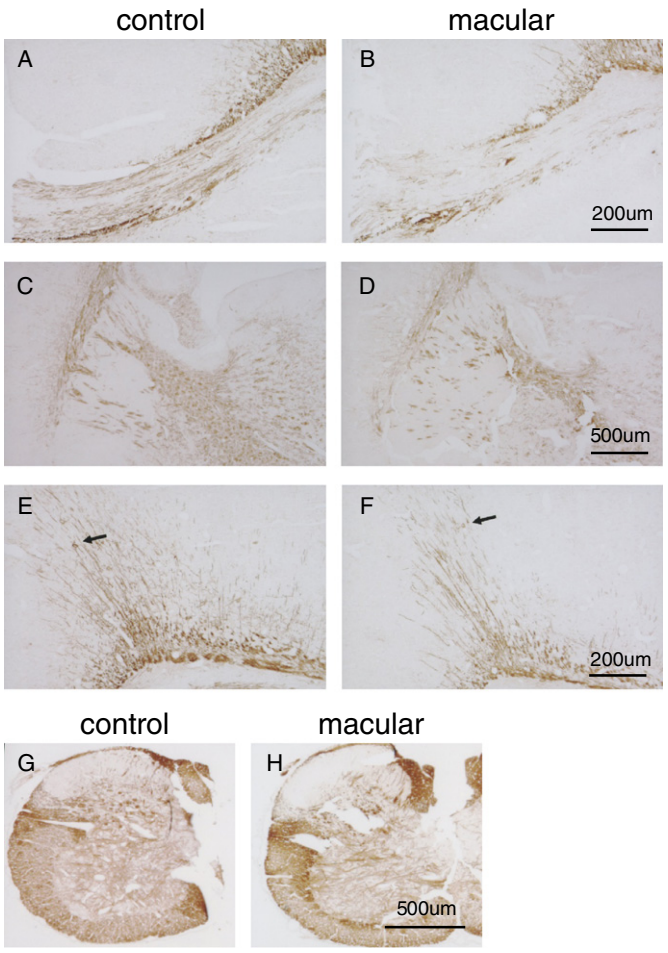

Fig. 1. CNPase immunohistochemistry. Representative images from corpus callosum (A and B), internal capsule (C and D), cingulate cortex (E and F) and spinal cord (G and H). The left panel contains images from control mice (A, C, E and G) and the right panel contains images from macular mice (B, D, F and H). Macular mice widely show hypomyelination in cerebral white matter as well as deep cortical layers. Hypomyelinated corpus callosum is observed in macular mice (A and B). Despite the scarcity of myelin sheath, myelin debris or myelin phagocytosis is absent. Myelin staining is weak and narrowly distributed within external and internal capsules of macular mice (C and D). Note that much fewer CNPase positive myelin sheaths are detectable in cingulate cortex of macular mice (E and F). Arrows indicate myelinating oligodendrocytes with CNPase immunoreactivity. In contrast, no obvious changes in distribution and immunoreactivity of CNPase are observed in white and gray matter of spinal cord (G and H). Scale bar: 200 μm (A, B, E and F), 500 μm (C, D, G and H).

cortex is not myelinated yet at PND 14, deep cortical layers of cingulate cortex are already myelinated by this developmental period. In macular mouse brain, the extent of myelination in cingulate cortex was also hypoplastic, similar to cerebral white matter (Fig. 1 E and F). In contrast, the extent of myelination in spinal cord of macular mice was comparable with controls (Fig. 1 G and H). Myelin debris or infiltration of inflammatory cells was not observed in hypomyelinated white matters using CNPase immunohistochemistry.

3.2. Increased apoptotic cells in macular mouse brain

Using cleaved caspase-3 immunohistochemistry, caspase-3 positive cells were occasionally seen in PND 14 controls. Apoptotic cell death plays an important role in eliminating neuronal cells to form proper neural circuits in developing brain [19]. Immature oligodendrocytes have also been reported to show apoptotic cell death during the myelination period [20]. However, macular mice contain far more caspase-3 positive cells than controls. As shown in Fig. 2, caspase-3 positive cells were increased in subcortical white matter, internal capsule,

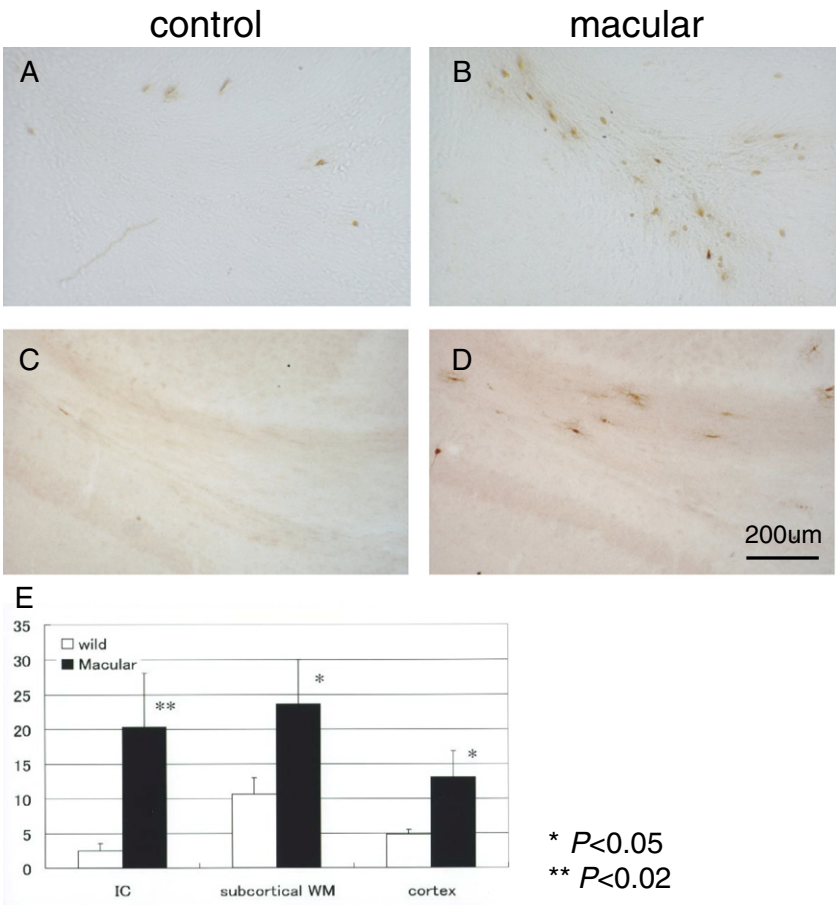

Fig. 2. Cleaved caspase-3 immunohistochemistry. Representative images of internal capsule (A and B) and corpus callosum (C and D). The left panel contains images from control mice (A and C) and the right panel contains images from macular mice (B and D). In controls, immunoreactive small cells were occasionally found in cerebrum. In macular mice, increased number of positive cells in internal capsule (A and B) and corpus callosum (C and D) were observed. Although caspase-3 positive cells were also increased in cerebral cortex, they were polymorphic and might include apoptotic neuronal cells other than oligodendrocyte lineage cells. Scale bar: 200 μm. Panel E shows quantitation of cleaved caspase-3 positive cells in internal capsule (IC), subcortical white matter (WM) including corpus callosum, and cerebral cortex. All areas show statistical differences between control and macular mice.

and cerebral cortex in macular mouse brain. Caspase-3 positive cells tend to be rich in hypomyelinated white matter, especially in corpus callosum and internal capsule (Fig. 2 A–D).

Although caspase-3 positive cells are polymorphic, these cells in white matter are morphologically similar to immature oligodendrocytes. Some caspase-3 positive cells have many processes with ellipsoid cytoplasm, and others have a spindle shape with relatively large nuclei (Fig. 3 A and B). These two types of cells were also observed in O4 immunoreactive cells. O4 is expressed by the late progenitor of oligodendrocytes and pro-oligodendrocytes as well as differentiated oligodendrocytes during rodent brain development in vivo [21]. Most O4 positive cells in control cerebral white matter show either of these two morphological types (Fig. 3 C and D). Although astro-gliosis is observed in macular brain, GFAP positive astrocytes (Fig. 3 E) are morphologically distinct from caspase-3 positive cells. Taken together, caspase-3 positive cells in white matter of macular mouse have highly similar morphology with O4 positive immature oligodendrocytes in control white matter.

4. Discussion

Using CNPase immunohistochemistry, hypomyelination in cerebrum (internal capsule, corpus callosum, and cingulate cortex) and relatively preserved myelination in spinal cord have been observed in macular mutant mouse. Hypomyelination of optic nerve has also been reported by ultrastructural evaluation in macular mouse brain [17]. In addition, decreased CNPase expression in macular mouse brain has been reported and thus hypomyelination was suggested biochemically [8,18]. However, a thorough histological evaluation of myelination of cerebral white matter of macular mouse has been lacking. Because of copper deficiency, such as Swayback disease in lambs, in which hypomyelination is induced [12], the pathogenesis of hypomyelination could be related to the level of copper. Copper plays an important role in cellular function through activities of various cuproenzymes. Cytochrome c oxidase activity has also been reported to be reduced in macular mouse brain [8,18]. Decreased cytochrome c oxidase activity suggests that mitochondrial dysfunction might be involved in hypomyelination through disrupted function of oligodendrocytes.

On the other hand, cuproenzymes, including cytochrome c oxidase and superoxide dismutase, contribute to the apoptotic cell death pathway through mitochondrial dysfunction or oxidative stress [22,23]. Because oligodendroglial apoptosis has been reported in various myelin disorders including multiple sclerosis [24], cell death of oligodendrocytes could be related to subsequent hypomyelination. Although neuronal apoptosis has been reported in cerebral cortex and hippocampus of macular mouse [10], evaluation of cell death of oligodendrocytes is lacking. We found numerous cleaved caspase-3 positive cells both in cerebral cortex and white matter. Caspase-3 is activated in apoptotic cells and cleaved caspase-3 antibody is used as a marker of apoptotic cell death. Although neuronal apoptosis is reported in gray matter, we have also observed increased TUNEL positive cells in cerebral white matter of macular mouse (data not shown). Those cells are assumed to be non-neuronal cells owing to their localization. Morphologically, these cells are distinct from GFAP positive astrocytes and resemble

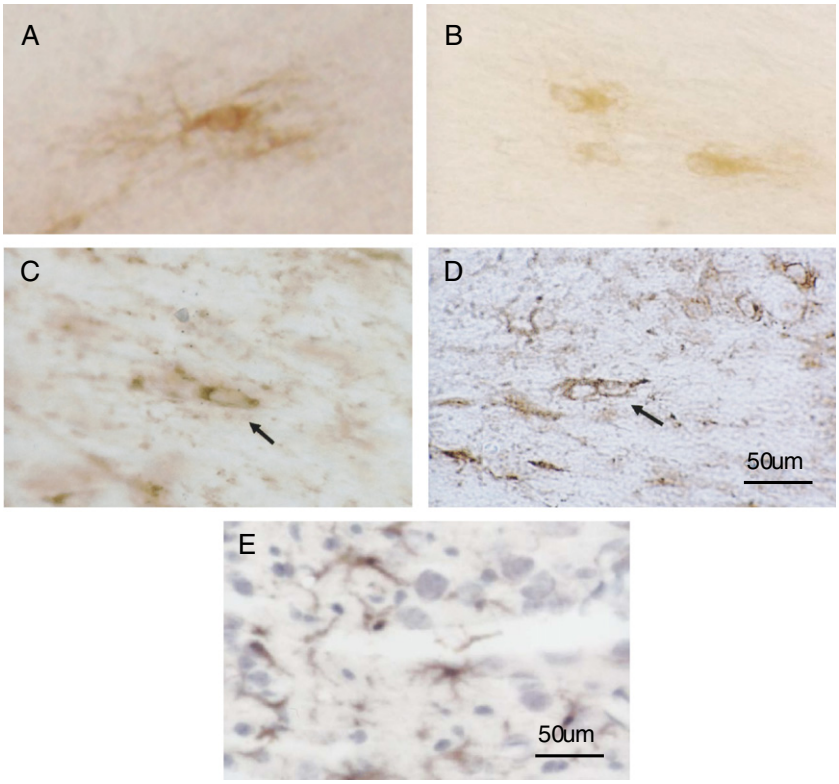

Fig. 3. Morphological features of cleaved caspase-3 positive cells and O4 positive immature oligodendrocytes. Representative images of cleaved caspse-3 (A and B), O4 (C and D), and GFAP (E). Immunoreactivity of caspase-3 in white matter of macular mice is mainly observed in two types of cells, cells with elliptic shape and abundant processes (A), or cells with spindle shape and relatively large nucleus (B). These morphological features are similar to those of premyelinating oligodendrocytes or oligodendrocyte precursor cells, respectively. O4 is known to be a marker of immature oligodendrocytes or oligodendrocyte precursor cells. O4 immunoreactivity visualizes round cell bodies of positive cells as well as their processes. O4 positive cells in control cerebral white matter show similar morphology with cleaved caspase-3 positive cells (arrows in C and D) in macular mice. For morphological comparison with caspase-3 positive cells, GFAP immunostaining is shown to demonstrate astrocytes in macular mice as shown in panel E. Scale bar: 50 μm (A–E).

immature oligodendrocytes or oligodendrocyte precursor cells labeled with anti-O4 antibody. Although double immune-labeling method is preferable, cleaved caspase-3 and O4 double labeling using fluorescent laser confocal microscopy is technically difficult on 50 μm thick vibratome sections. Because we have found no debris or phagocytosis of myelin in CNPase immunohistochemistry, mature- myelinating oligodendrocytes are assumed to be intact in our study. Therefore, cleaved caspase-3 positive cells in cerebral white matter could be degenerated immature oligodendrocytes or oligodendrocyte precursor cells. Because spinal cord receives myelination earlier than cerebrum, relatively preserved myelination in spinal cord of macular mouse and information about susceptibility of developing oligodendrocytes from literature [25] imply that mature oligodendrocytes are less susceptible to injury due to copper deficiency.

Other possibility of hypomyelination can be a direct consequence from *ATP7A* deficiency regardless of cupper content. Several literatures explore the role of ATP7A protein in developing brain [26,27]. Meskini et al. reported the role of ATP7A protein in developmental axon outgrowth which is possibly independent of decreased cupper level in brain [28,29]. However, Ohno et al. reported that cupper deficiency is a key factor triggering neuronal apoptosis in macular mouse by showing no increase of apoptotic cells in the brains of copper-supplemented hemizygotes [10]. Although it is possible that hypomyelination in macular mutant mouse is independent of cupper deficiency and is rather due to deficient ATP7A itself, this possibility remains to be elucidated.

An in vitro study has shown that administration of cuprizone to oligodendrocyte-enriched cultures resulted in inhibited oligodendrocyte maturation [15]. Cuprizone is known to induce demyelination in weaning rodents through its ability to chelate copper. Our report indicates that copper deficiency would affect immature oligodendrocytes and cause degeneration of these cells. Trapp et al. reported that immature-premyelinating oligodendrocytes turn apoptotic when their maturation is interrupted [20]. Given that decreased activity of mitochondrial cuproenzymes and mitochondrial abnormality in animal models of Menkes disease are reported [8,11], we speculate that copper deficiency might affect immature oligodendrocytes through decreased activity of cuproenzymes and cause perturbed maturation and apoptosis of these cells, leading to subsequent hypomyelination.

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