Absence of mechanical allodynia and Aβ-fiber sprouting after sciatic nerve injury in mice lacking membrane-type 5 matrix metalloproteinase

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Abstract Matrix metalloproteinases (MMPs) are a family of endopeptidases that degrade extracellular matrix components. Membrane-type 5 MMP (MT5-MMP/MMP-24) was identified as neuron-specific, and is believed to contribute to neuronal circuit formation and plasticity. To elucidate its function in vivo, we have generated mice lacking MT5-MMP by gene targeting. MT5-MMP-deficient mice were born without obvious morphological abnormalities. No apparent histological defects were observed in the nervous system either. However, MT5-MMP-deficient mice did not develop neuropathic pain with mechanical allodynia after sciatic nerve injury, though responses to acute noxious stimuli were normal. Neuropathic pain induced by peripheral nerve lesions is known to accompany structural reorganization of the nervous system. Intraneural injection of cholera toxin B subunit, a transganglionic tracer, into the injured sciatic nerve of wild-type mice revealed that the myelinated Aβ-fiber primary afferents sprouted from laminae III–VI of the dorsal horn of the spinal cord and invaded lamina II. However, no such sprouting and invasion of Aβ-fibers were observed in MT5-MMP-deficient mice. These findings suggest that MT5-MMP is essential for the development of mechanical allodynia and plays an important role in neuronal plasticity in this mouse model.

Key words: Matrix metalloproteinase; Membrane-type matrix metalloproteinase; Gene targeting; Mechanical allodynia; Sprouting

1. Introduction

Pericellular proteolysis is an important step in the regulation of various cellular functions in tissue through proteolytic conversion of the proteins in the extracellular milieu [1,2]. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are responsible for degradation and processing of protein components in the extracellular matrix (ECM) and cell surface molecules including cell adhesion molecules, cytokines, growth factors, and receptors [3,4]. Because of this activity, MMPs have been implicated not only in a wide array of physiological processes [5], but also in diverse pathological states [3,6]. Although most MMPs are secreted enzymes, six are membrane-type MMPs (MT-MMPs) having either a type I transmembrane domain (MT1-, MT2-, MT3- and MT5-MMP) or a glycosylphosphatidylinositol-anchor (MT4- and MT6-MMP) [7]. These MT-MMPs are particularly suitable for the pericellular proteolysis that regulates cellular functions.

MT5-MMP (MMP-24) was identified as a brain-specific MMP [8,9] and its expression was specifically detected in neurons of both the central and peripheral nervous systems [10,11]. During embryonic and early postnatal development in rodents, MT5-MMP is abundantly expressed throughout the nervous system [10,12]. Subsequently in adults, significant expression levels are maintained in regions of neuronal plasticity, such as the cerebellum and hippocampus [10–12]. The temporal and spatial regulation of the expression suggests that MT5-MMP plays roles in neuronal circuit formation and plasticity. To gain further information on the roles of MT5-MMP in vivo, we generated MT5-MMP-deficient mice by targeted disruption of the gene (*mmp-24*).

2. Materials and methods

2.1. Generation of MT5-MMP-deficient mice

Genomic DNA fragments were obtained by screening a mouse 129/SvJ genomic library in lambda FIX II (Stratagene). A targeted construct was designed to replace the 3.3-kb genomic fragment of *mmp-24* containing exon 5 and part of exon 6 with a MC1-neo cassette (*neo*). The 5′ 4.3-kb (*Xba*I–*Hin*dIII fragment) and 3′ 5.5-kb (*Bam*HI–*Cla*I fragment) fragments were used as sites of homologous recombination between the vector and the genomic sequences (Fig. 1). The diphtheria toxin A gene regulated by the MC1 promoter (*DT-A*) was added to the 3′ end. The targeting vector was linearized at the *Xba*I site and introduced into the embryonic stem (ES) cell line E14-1 by electroporation. The cells were selected in medium containing G418 (400 μg ml^{-1}; Invitrogen) and drug-resistant clones were tested for homologous recombination by Southern blot analysis with specific probes. Three clones containing the mutated *mmp-24* allele were identified and Southern blot analysis was performed further with the *neo* probe to confirm the single targeting event. Cells from two independent targeted clones were microinjected into C57BL/6J blastocysts and chimeric male founders were obtained. To obtain MT5-MMP-deficient mice on an inbred congenic genetic background, the heterozygous mice obtained were backcrossed to strain C57BL/6J for at least eight generations. These heterozygous mice were then intercrossed to generate homozygous mice.

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Abbreviations: MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; ECM, extracellular matrix; CTB, cholera toxin B subunit; ES, embryonic stem; CSPG, chondroitin sulfate proteoglycan

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2.2. Neuropathic pain model

To generate a neuropathic pain that mimics mechanical allodynia in mice, the sciatic nerve was injured by ligation or transection as described in previous studies [13,14] and the response to a mechanical stimulus was measured. Mice used for the experiment were prepared by crossing the heterozygous mice, but the observer was blind to the genotype throughout the experiment. Twelve-week-old mice were anesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.). The right sciatic nerve was exposed at mid-thigh level and tightly ligated with 8–0 silk suture around approximately 1/3 to 1/2 the diameter of the nerve or transected. As a control, the left sciatic nerve was subjected to a sham operation with the same surgical procedure without nerve injury. Before the test to measure the response to the mechanical stimuli, mice were placed in a transparent plastic box with a metal mesh floor and allowed to acclimate for 15 min. The mid-planter surface of the hind paw was stimulated using von Frey filaments (North Coast Medical, Inc.) for 6–8 s with increasing force. The force of stimuli that cause a brisk withdrawal in 50% of the trials was calculated by using the up–down method of Dixon, as described previously [15], and presented as the 50% withdrawal threshold. The values were expressed as the mean ± S.E.M. Mechanical allodynia can be observed as a decrease in the threshold to the stimuli. All the experiments were performed in accordance with the guidelines presented by the Ethics Committee of the International Association for the Study of Pain [16].

2.3. Specific labeling of Aβ-fibers using cholera toxin B subunit (CTB) conjugated with horseradish peroxidase (CTB-HRP)

Two weeks after the surgical operation, mice were anesthetized and the sciatic nerves on both sides were re-exposed. Two μl of a 1.5% solution of CTB-HRP was injected into the proximal site of the injured sciatic nerve using a 33G needle and Hamilton microsyringe. Three days after the CTB-HRP injection, the animals were anesthetized and the fourth and fifth lumbar (L4 and L5) segments were dissected out. Following a rinse with normal saline, the specimens were immersed and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4°C and stored overnight in 30% sucrose in 0.1 M phosphate buffer at 4°C. Fifty-μm-thick transverse sections of spinal cord were cut and were processed for HRP histochemistry using tetramethylbenzidine as the chromogen [17].

3. Results and discussion

3.1. Generation of MT5-MMP-deficient mice

To disrupt the *mmp-24* gene in mice, the 3.3-kb genomic fragment containing exon 5 and part of exon 6 was substituted with a segment containing a drug-resistant neo gene (MC1-*neo* cassette) in ES cells (Fig. 1A). The deletion results in a loss of the zinc-binding site in the catalytic domain, which is essential for the enzyme activity, and a frame shift at the junction between exons 4 and 7 in the final mRNA product. Thus, the mutated gene encodes only a propeptide sequence with the residual N-terminal part of the catalytic fragment. Heterozygous mice having the mutated *mmp-24* allele were obtained with no obvious defects and were interbred to generate homozygous mice. To determine the genotypes of the progeny, genomic DNA was obtained and analyzed by Southern blotting using the 5′ probe and 3′ probes after digestion with *Ssp*I or *Sac*I (Fig. 1B). To examine the expression of the mutated gene, Northern blot analysis of total RNA derived from neonatal whole brain was performed (Fig. 1C). A 4.5-kb mRNA band was detected in the wild-type and the heterozygous mice, but it was absent in the homozygous mice. A faint band at 4.1 kb that is presumably derived from the mutated gene appeared in the heterozygous and homozygous mice.

MT5-MMP-deficient mice were born in the predicted Mendelian ratio (29.7% wild-type, 44.8% heterozygous, and 25.3% homozygous, $n = 205$) following intercrosses between heterozygous mice. The null mice have a normal appearance, fertility, and lifespan. General behavioral analysis demonstrated no significant difference between the wild-type and null mice except for the following. In the rotarod test to assess motor abilities, mice were placed on the rotating drum individually and the number of falls in a 3-min period was counted. MT5-MMP-deficient mice exhibited significantly less falls than the wild-type mice (7 ± 0.7 versus 2 ± 0.3 (5 week old) and 2 ± 0.4 versus 0.2 ± 0.1 (8 week old), $P < 0.01$, Student's *t*-test). In the water T-maze test to evaluate learning ability, the null mice did not try to swim by just floating on the water. However, they started to swim like wild-type mice when they were picked by an observer. Thus, MT5-MMP deficient mice may have defect to feel the stress in water. Histologic examination of sections from the nervous tissues including brain, spinal cord and dorsal root ganglion did not demonstrate obvious abnormalities in the null mice (data not shown).

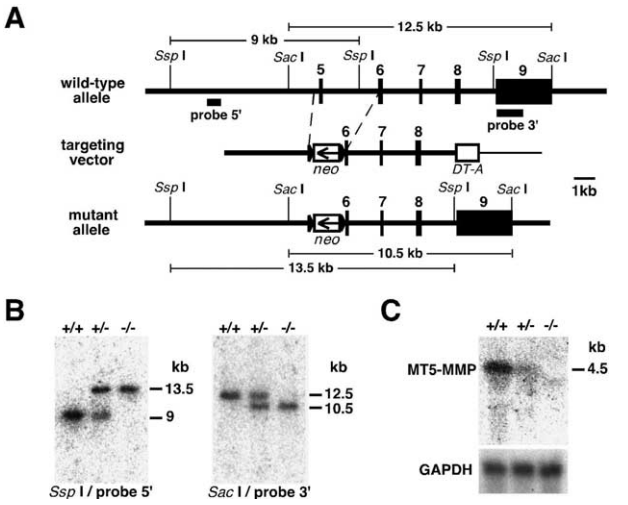

Fig. 1. Gene targeting of MT5-MMP in the mouse. A: Targeting strategy. The relative positions of exons 5–9 (solid boxes) are indicated. The targeting vector contains a MC1-neo cassette (*neo*) flanked by loxP (arrowheads) and a diphtheria toxin A gene driven by the MC1 promoter (*DT-A*). Arrows indicate the transcriptional direction. The positions of the external 5′ and 3′ probe used for delineation of the target event are illustrated. B: Southern blot analysis of mouse tail DNA isolated from the progeny of a mating between heterozygous parents. C: Northern blot analysis of total RNA from whole brains of neonatal mice.

3.2. Absence of mechanical allodynia after sciatic nerve injury in MT5-MMP-deficient mice

MT5-MMP-deficient mice did not show any obvious defects under physiological conditions. Then, we examined the effect of the MT5-MMP deficiency on the development of a severe, long-lasting neuropathic pain caused by lesions in the nervous system [18]. One characteristic feature of neuropathic pain is mechanical allodynia, which is defined as pain evoked by non-noxious mechanical stimuli. Clinically, no adequate therapies are available for neuropathic pain and, therefore, several experimental animal models have been developed to study the mechanisms involved [13,14,19,20]. Peripheral nerve injury in animals is known to cause this type of chronic pain even after complete healing of the injured tissues and also induces structural reorganization of the terminal nerve fibers in the dorsal horn spinal cord based on the plasticity of the injured neurons [21–23].

To examine the potential involvement of MT5-MMP in the

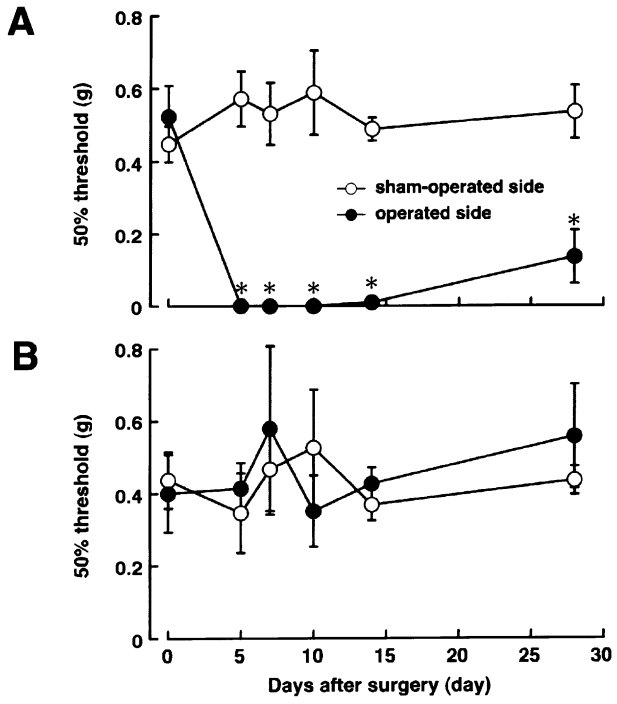

Fig. 2. Assessment of mechanical sensitivity after sciatic nerve injury in wild-type and MT5-MMP-deficient mice. A: In wild-type mice, paw withdrawal thresholds (presented as 50% threshold) following mechanical stimulation for the injured side (filled circles) showed a significant decrease compared with the sham-operated side (open circles). B: In MT5-MMP-deficient mice, no reduction in mechanical sensitivity was observed throughout the 28-day study period. Asterisks indicate $P < 0.01$ (Mann–Whitney U-test); $n = 9$ for wild-type and $n = 6$ for MT5-MMP-deficient mice.

mouse neuropathic pain model, the sciatic nerve on the right side was partially ligated and paw withdrawal in response to mechanical stimuli added to the mid-planter surface of the hind paw was monitored. The force of stimuli that caused a withdrawal response in 50% of trials (50% threshold) is indicated in Fig. 2. Withdrawal response in the sham-operated left side was measured as a control. In the wild-type mice, the threshold of the response to the stimuli decreased significantly and persisted for at least 28 days (Fig. 2A). Basically the same result was obtained when the sciatic nerve was injured by transection (data not shown). In contrast, MT5-MMP-deficient mice did not show a decrease in the threshold value of the response to mechanical stimuli (Fig. 2B). Thus, MT5-MMP looks indispensable to the occurrence of mechanical allodynia after peripheral nerve injury.

However, there is a possibility that the absence of neuropathic pain in the MT5-MMP-deficient mice is a result of reduced transmission of pain sensation. We think this is not the case, because no difference was observed in the paw withdrawal responses between wild-type and MT5-MMP-deficient mice on the sham-operated side. In addition, responses of MT5-MMP-deficient mice to various noxious stimuli, such as heat, formalin, and acetic acid, appeared normal compared to the wild-type mice (data not shown).

3.3. *Transganglionic labeling of the central terminals of primary afferents in the dorsal horn of the spinal cord*

Previous studies have demonstrated that peripheral nerve lesions induce neuronal plasticity with structural reorganization [21–23]. The central termination pattern of primary afferents has been found to change profoundly in the dorsal horn of the spinal cord following peripheral nerve damage in rat. Myelinated Aβ-fibers, which only elicit innocuous sensations, terminate in laminae III–VI in the dorsal horn (Fig. 3). Within 2 weeks after peripheral nerve injury, Aβ-fibers sprout from laminae III–VI and invade lamina II where unmyelinated C fibers terminate and nociceptive-specific neurons are dominant (Fig. 3). This structural reorganization is believed to be important for the sustained neuropathic pain. In addition, the Aβ-fibers form synapse-like structures in lamina II [23–25] and novel mono- or polysynaptic Aβ fiber-mediated inputs can be detected there [26–28]. These results suggest that the extended Aβ-fibers transmit an innocuous coetaneous sensation to neurons of the superficial dorsal horn that normally receive a nociceptive input. Thus, the observed structural reorganization is considered to be important for the sustained neuropathic pain with mechanical allodynia.

MT5-MMP is expressed in isolated dorsal root ganglion neurons [10] that express a myelinated A-fiber marker, 200-kDa neurofilaments (NF200) [29]. In addition, MT5-MMP was particularly localized to the growth cone, the leading structure of growing axons. Thus, MT5-MMP-deficient mice may be defective in the sprouting and invasion by Aβ-fibers in the dorsal horn. To examine this possibility, we used CTB which binds to the GM1 ganglioside present on myelinated A-fibers but not on unmyelinated C-fibers as a retrograde and transganglionic tracer [29]. CTB-HRP was injected into the proximal site of the damaged sciatic nerve and its transport to the dorsal horn of the spinal cord was monitored (Fig. 3).

When CTB-HRP was injected into the sham-operated sites of both wild-type and MT5-MMP-deficient mice, specific labeling of laminae III–VI in the spinal dorsal horn was observed and lamina II was not stained at all (Fig. 4A,C). This staining pattern is consistent with previous studies [22,25,30]. When CTB-HRP was injected into the injured side of wild-type mice, extended labeling of lamina II was observed (Fig.

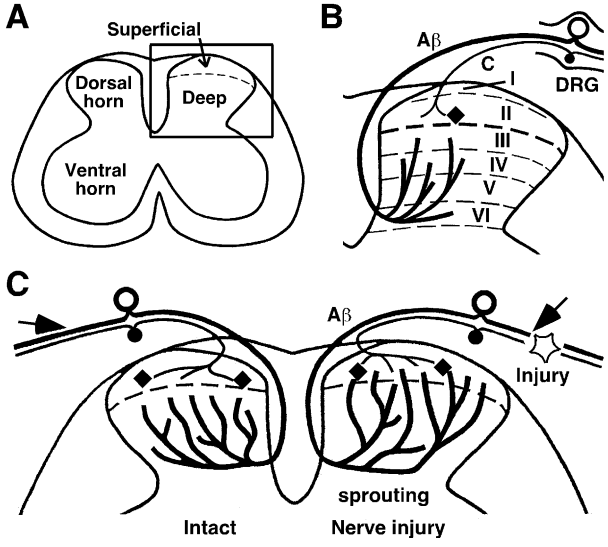

Fig. 3. A: Schematic diagram of the spinal cord at L4. B: Boxed area of A. I–VI, cytoarchitectonic laminae; DRG, dorsal root ganglion; Aβ, myelinated Aβ-fibers; C, unmyelinated C-fibers. A filled square indicates the cell body of the nociceptive-specific neuron. C: After peripheral nerve injury, Aβ-fibers sprout from laminae III–VI into lamina II. Arrows indicate the injection site of CTB.

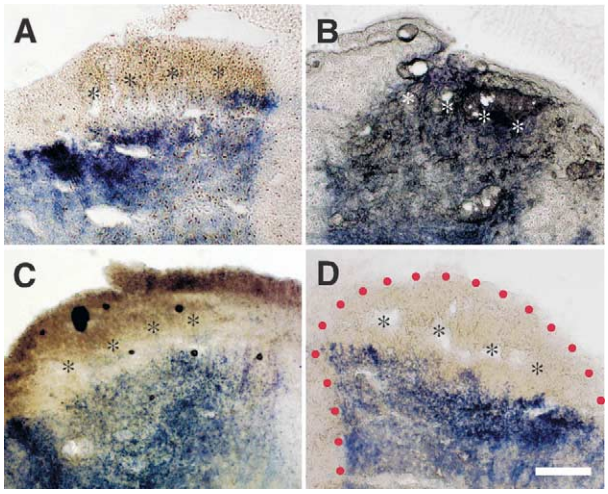

Fig. 4. CTB-HRP labeling in dorsal horn lamina II following sciatic nerve transection. CTB-HRP is bilaterally injected into the sciatic nerve 14 days after sciatic nerve transection and examined in the dorsal horn in L4–L5 segments of spinal cord 17 days after axotomy. Sham-operated side in wild-type mice (A) and MT5-MMP-deficient mice (C). Note that lamina II (asterisks) in each genotype is devoid of labeling. Injured side in wild-type mice (B) and MT5-MMP-deficient mice (D). In wild-type mice, CTB-HRP label is present in lamina II as well as the other laminae. In contrast, lamina II in MT5-MMP-deficient mice is completely devoid of labeling as observed on the sham-operated side. Scale bar = 100 μm.

4B) as in the previous studies [21,22,25]. In contrast, CTB-HRP staining in lamina II was clearly absent on the injured side of MT5-MMP-deficient mice (Fig. 4D). Thus, MT5-MMP is important in the sprouting and invasion by Aβ-fibers into lamina II after sciatic nerve injury.

Neurons are reported to express multiple MMPs and have been suggested to play roles in the remodeling of surrounding ECM components [31,32]. However, their in vivo function in the nervous system has remained to be elucidated. Thus, this is the first demonstration that one of the MMPs expressed in neurons plays a critical role in neuronal plasticity accompanying the structural remodeling of the nervous system. However, the critical substrates that have to be degraded by MT5-MMP during the process remain to be studied. Previously, MT5-MMP was reported to degrade the core proteins of chondroitin sulfate proteoglycans (CSPGs) [33] that are abundant in neuronal tissues [34]. Since CSPGs are inhibitory to neurite outgrowth [35,36], they are possible targets of MT5-MMP in the tissue.

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