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ORIGINAL

Metallothionein contributes to neuropathic pain in partial sciatic nerve ligated rats

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ABSTRACT

Neuropathic pain is a chronic pain state caused by nerve injury or diseases. The symptoms involve spontaneous pain, hyperalgesia and allodynia. Neuropathic pain develops by the mechanisms both central nervous system and peripheral nervous system. Moreover, both neuronal cells and glia cells are involved in the development of neuropathic pain. However, the pathogenic mechanism of neuropathic pain is not clearly understood. We previously reported that metallothionein lacked in peripheral nerve from patients of complex regional pain syndrome by proteomic approach. In this report, we examined whether the level of metallothionein (MT) is changed in partial sciatic nerve ligation (PSL) rats as the model animal of neuropathic pain and the administration of metallothionein affects behavior against physical and thermal stimulus to PSL rats. MT-I/II expression was gradually decreased in the distal region of the injury site. At day 28, MT-I/II expression was markedly decreased in both proximal and distal region at the same level. The administration of MT significantly improved allodynia and thermal hyperalgesia comparing to the administration of PBS. Moreover, GAP43, a marker protein of nerve regeneration, increased in the distal region and g lial fibrillar acidic protein, a marker protein of inflammation, decreased in the proximal region of the injury site. These results suggest that metallothionein is deeply related to occurrence of neuropathic pain and regeneration of the injured nerve in PSL rats.

Key words: metallothionein, neuropathic pain, allodynia, oxidative stress, reactive oxidative species

1. Introduction

Neuropathic pain is a chronic pain state caused by nerve injury or diseases such as cancer or diabetes[10,41]. The typical symptoms of neuropathic pain involve spontaneous pain, hyperalgesia and allodynia. Neuropathic pain develops by the mechanisms of both central (CNS) and peripheral nervous systems (PNS)[10]. For instance, much preclinical evidence indicates that N-methyl-D-aspartate receptors (NMDARs) are crucial to pain hypersensitivity[29]. On the other hand, the expression of multiple sodium channels altered dramatically in dorsal root ganglion (DRG) neurons after axotomy[45]. This alteration is accompanied by electrophysiological changes that poise DRG neurons to fire spontaneously or at inappropriate high frequencies. In addition to the neuronal pathway, glia cells such as Schwann cells and microglia cells, and immune cells are involved in the development of neuropathic pain. After injury of peripheral nerve, activated spinal microglia contribute to the pathologically enhanced pain processing in the dorsal horn that underlies neuropathic pain[41,44]. Moreover, proinflammatory cytokines released from Schwann cells and immune cells, such as macrophages and mast cells, modulate to spontaneous nociceptor activity and stimulus sensitivity[41]. However, the pathogenic mechanism of neuropathic pain is not clearly understood.

We previously revealed that metallothionein (MT)-I/II defected in the injured peripheral nerve from the patients with complex regional pain syndrome (CRPS)-2 and painful neuroma using a proteomics approach[33]. CRPS is a chronic pain disorder tipically displayed neuropathic pain. MTs are low molecular weight (6-7 kDa) proteins with abundant cysteine residues (close to 30% of all amino acid components). In mammals, MTs are divided into four

subfamilies (MT-I, II, III and IV). MT-I and MT-II are widely expressed and regulated coordinately, whereas MT-III and MT-IV are expressed predominantly in the CNS and squamous epithelia, respectively[12]. The MT-I and MT-II isoforms are remarkably similar, so they are often discussed as a single isoform (MT-I/II) [18]. MTs bind preferentially to heavy metals, including both xenobiotic and physiologic substances. This property is thought to be important in protecting organisms against metal toxicity especially cadmium[4]. MTs are multifunctional proteins induced by various chemicals and cytokines such as glucocorticoids, interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNFα) and reactive oxygen species (ROS) [34]. Their major functions are thought to maintain the homeostasis of intravital heavy metals, defense against oxidative stress and scavenge for ROS. There are some reports which show the usefulness for the treatment of CNS diseases such as autoimmune encephalomyelitis, forcal cortical brain injury and Parkinson disease[11,16,35].

The purpose of this study is to evaluate the roles of MT in injured nerves of partial sciatic nerve ligation (PSL) rats. We examined the levels of endogenous MT in injured nerves and the effectiveness of ecombinant MT to painful behaivior in PSL rats.

2. Materials and Methods

2.1. Animals

All procedures were approved by the Institute of Animal Experiment at Sapporo Medical University and complied with the recommendations of the International Association for the Study of Pain. We used female Sprague-Dawley rats weighing 240-280 g in all experiments (SLC, Tokyo, Japan). PSL was performed according to the method previously described[42]. In brief, the sciatic nerve (SCN) of the right hindlimb was tightly ligated with a 8-0 nylon suture at the dorsal one half of the nerve thickness in the high thigh.

2.2. Behaviral study

PSL-induced tactile allodynia and thermal hyperalgesia were assessed by the von Frey test and the Hargreaves test, respectively, as described previously[31]. Briefly, in the von Frey test, the mechanical withdrawal response was measured as the frequency of withdrawals of the hind paw of rats elicited by a defined mechanical stimulus of 1.26 g, 4.35 g and 8.75 g using a calibrated nylon filament (Semmes-Weinstein Monofilaments; North Coast Medical, San Jose, CA). The right (constriction side) and left (contralateral side) paw was probed consecutively by ten tactile stimulations alternately. Each test was repeated three times which resulted in each foot receiving 30 mechanical stimulations. In the Hargreaves test, the thermal withdrawal response was measured as the latency of withdrawals of the hind paw of rats elicited using Tail Flick Analgesia Meter (IITC life science, Woodland Hills, CA). A cut-off time of 10 seconds was set to prevent tissue damage. Each hind paw was tested five times, alternating between the left and the right. Consequently, the thermal withdrawal latency of of each rat was defined as the latency of the contralateral response (non-constricted) minus the ipsilateral response (constricted). Positive scores indicated increased and negative scores decreased sensitivity of the ipsilateral hind paw. The behavioral tests were performed on days 0 (before surgery), 1, 3, 5 and 7.

2.3. Immunological investigation

Rats were anesthetized and transcardially perfused with 4% paraformaldehyde phosphate buffered solution and 2 cm of SCN containing ligation site was removed and embedded in frozen sectioning media (FSC 22; Leica). Immunohistochemistry was performed according to the method previously described[33]. To enable an objective and quantitative scoring system, slides were then captured with a microscope (BX40F4, OLYMPUS, Japan) connected with a CCD camera (DP70, OLYMPUS, Japan) as digital images. Microscopic fields were analyzed per captured image at a magnification of x40[40]. These images were analyzed using Image J (Version 1.44) analysis software (National Institutes of Health, Bethesda, MD, USA). These images from three different sections of each nerve were converted to grayscale. These converted images are expressed in pixels. These levels of pixels were normalized against the background. The averages of normalized pixels of these images were calculated. For immunofluorescence microscopy, rats were anesthetized and transcardially perfused with 4% paraformaldehyde phosphate buffered solution and 2 cm of the SCN containing ligation site was removed and frozen. Tissue sections were then incubated in 1% BSA/PBS containing anti-GAP43 antibody (1:1000; Zymed) or anti-GFAP antibody (1:800; CHEMICON) for 30 min at room temperature. After being washed twice with PBS, tissue sections were incubated with the Alexa Fluor 488-conjugated secondary antibodies (Invitrogen). Tissue sections were examined using an inverted confocal laser scanning microscope (Zeiss LSM 510; Carl Zeiss).

2.4. Recombinant metallothionein protein

Total RNA was isolated from human sural nerve using TRI Reagent (SIGMA, Tokyo, Japan) according to the protocol of the manufacturer. Reverse transcription into first-strand DNA was achieved using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Tokyo, Japan) according to the instructions from the manufacturer. The full-length cDNA of human MT-IIA (hMT-IIA) was amplified by PCR from the first-strand DNA of human sural nerve using the following primers: 5'-GGAATTC ACTCTAGCCGCCTCTTCAG-3' and 5'-GGAATTC GTCGCGTTCTTTACATCTGG-3'. The PCR products were subcloned into pBluescript II SK (+) cloning vector (Agilent Technologies, Tokyo, Japan), and then sequenced. For subsequent subcloning into expression vectors, the full-length hMT-IIA cDNA was generated by PCR using primers as follows: a forward primer (5'-GAAGATCTA TGGATCCCAACTGCTCCTG-3'), and a reverse primer (5'-GGAATTCAGGCGCAGCAGCTGCACTTG-3'). Plasmids encoding the hMT-IIA fused to GST (glutathione S-transferase) were obtained by inserting the hMT-IIA cDNA into the pGEX-6P-1 vector (GE Healthcare, Tokyo, Japan), and transformed BL21 cells (Agilent Technologies). The GST-fusion proteins was induced by 0.1 mM isopropyl b-D-thiogalactoside at 30°C for 8 h. Cells were then lysed by sonication in lysis buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 0.25 M sucrose, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, complete protease inhibitor mixture (Roche)), and insoluble material was removed by centrifugation at 10,000 g for 30 min. The supernatants were incubated in batches with glutathione-Sepharose 4B (GE Healthcare) at 4°C for overnight, and beads were then washed three times with PBS. The beads were packed the column and then washed with the protease buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl). Recombinant hMT-IIA (rhMT-IIA) was purified by treating GST-hMT-IIA bound to beads with PreScission Protease (GE Healthcare) overnight at 4 °C and kept on -20 °C until the administration to PSL rats.

2.5. Aministration of rhMT-IIA to PSL rats.

The day of surgery, 20 μg rhMT-IIA in 100 μl PBS or PBS (as control) was injected directly in the SCN (intraneural injection). From day 1 to day 6, 10 μg rhMT-IIA in 400 μl PBS or PBS was injected around the SCN (perineural injection).

2.6. Statistical analysis

All the data are presented as the mean ± SEM. The statistical analysis for behaviral study was performed using split-plot design ANOVA. The statistical significance was set at $p<0.05$.

3. Results

3.1. Metallotionein expression of injured nerve in PSL rats

We recently revealed that MT-I/II defected in the injured nerve from the patients with CRPS-2 and painful neuroma[33]. This result suggested that expression of MT-I/II was correlated with pain. In the studies of neuropathic pain, various animal models were used. To evaluate the dynamic state of MT-I/II in model animals caused neuropathic pain, we investigated the level of MT-I/II in the sciatic nerve of PSL rat using immunohistochemistry (Fig. 1 and 2). At day 3, the expression level of MT-I/II was weakly decreased in the distal region of the injury site (Fig. 1E). Subsequently, MT-I/II expression gradually decreased in the distal region (Fig.1F, G and H). In the proximal region, however, MT-I/II almost unchanged until day 14 (Fig. 1E, F, G and H). At day 28, MT-I/II expression was markedly decreased in both proximal and distal region at the same level (Fig. 1H). The expression of MT-I/II in the ligation site decresased significantly from day 7 compared to proximal and distal site. At day 14, the expression of MT-I/II in the distal site decreased significantly conmapred to the proximal site. Finnaly, the expression of MT-I/II in the proximal site also degreased at day 28 (Fig. 1I). Theese results showed that the expression level of MT-I/II was decreased in the injured nerve of PSL rats as with the those in CRPS patients[33].

3.2. Eficacy of rhMT-IIA to PSL rats

Recently, it has been reported that the administration of MT improved neurodegenerative disorders in the CNS[16,17,20,36,37]. We administered rhMT-IIA protein to the PSL rats daily starting at the day of PSL sugery and examined behavioral tests (Fig. 3). The withdrawal response frequency was no difference between treatment group and control group until the day 3. But the number of withdrawal response in the treatment group decreased significantly after the day 5. Withdrawal latency from heat stimuli also decreased significantly in a treatment group at the day 7.

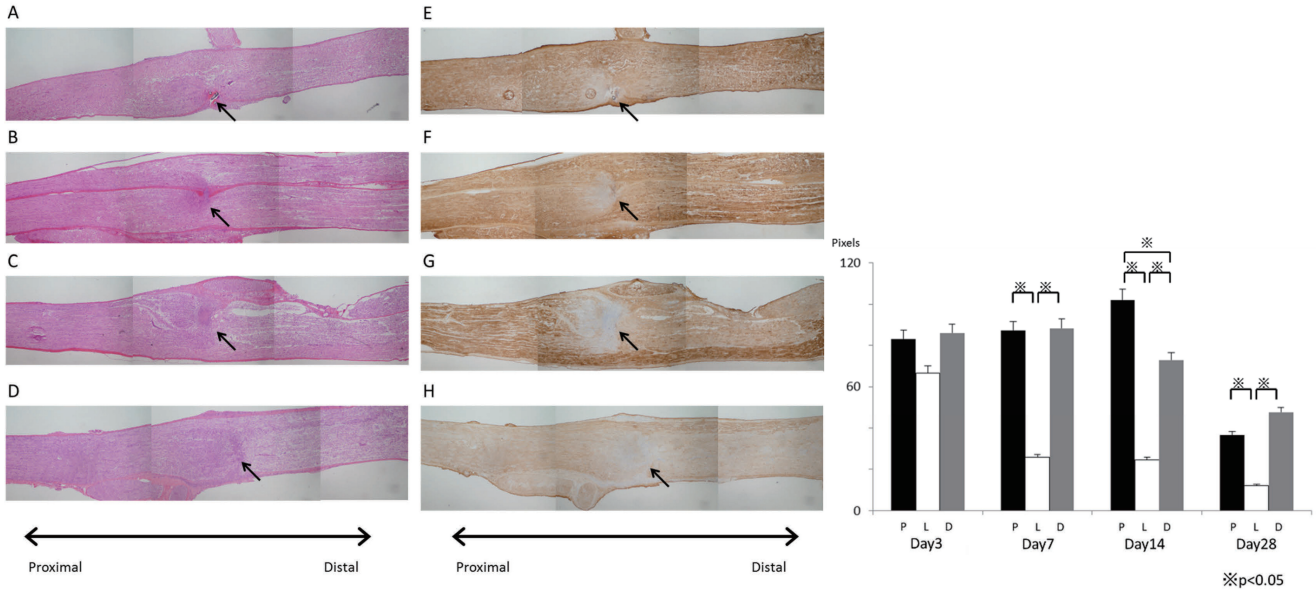

Fig. 1. Expression of MT in PSL rats. The injured nerve was resected at 3 (A, E), 7 (B, F), 14 (C, G) and 28 (D, H) days after ligation of sciatic nerve. Tissue sections were performed hematoxyline-eosin staining (A, B, C, D) and immunostaining with anti-MT (E, F, G, H). Black arrows indicate the ligation point. Magnification is 40x. Bar graph are shown as the mean ± SEM of 4 rats.

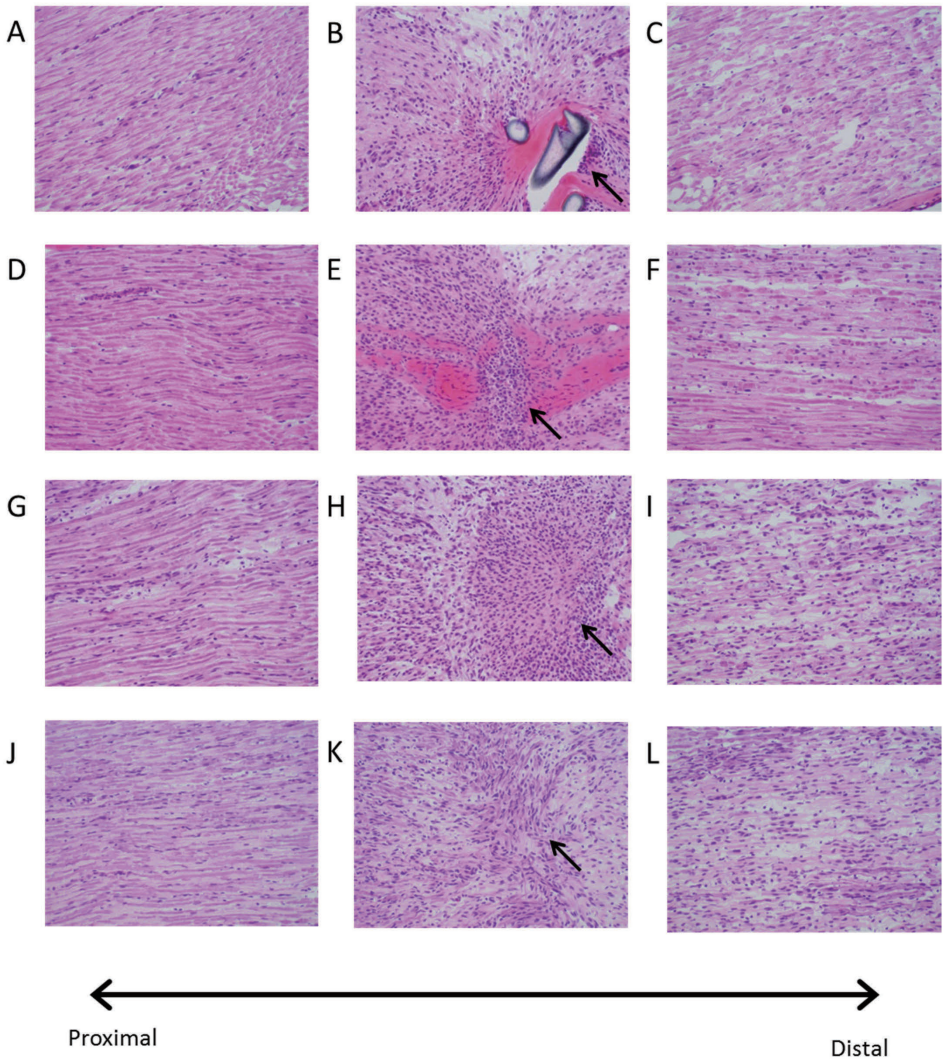

Fig. 2. Sections of the sciatic nerve from PSL model rats. SCN was resected at 3 (A, B, C), 7 (D, E, F), 14 (G, H, I) and 28 (J, K, L) days after ligation. All these were preformed hematoxyline-eosin staining. Black arrows indicate the ligation point. Magnification is 100x.

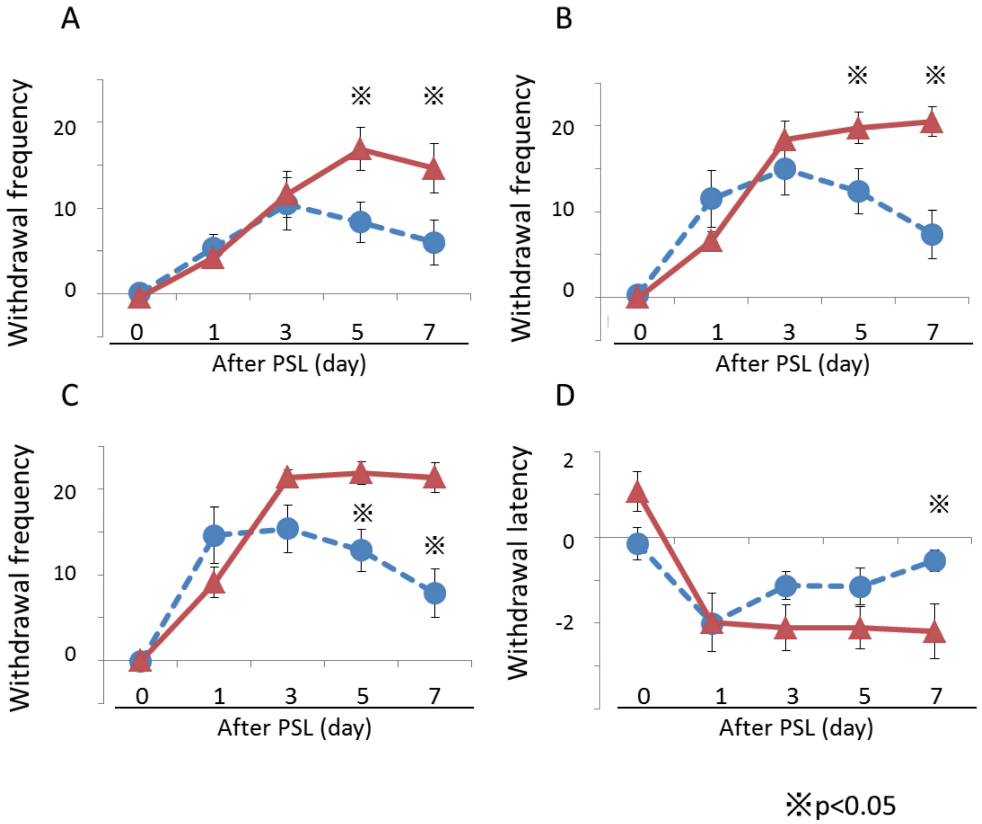

Fig. 3. Effect of recombinant MT on tactile allodynia and thermal hyperalgesia in PSL rats. The rhMT-IIA protein (circle) or PBS (triangle) was injected as described in "Materials and Methods". The von Frey test (A; 1.26g, B; 4.35g, C; 8.75g) and the Hargreaves test (D) were performed on days 0, 1, 3, 5 and 7. Data are the mean ± SEM from experiments using 8 rats. *p<0.05 as compared with PBS at the same time.

3.3. Morphology of injured SCN with or without rhMT-IIA

Comparing the SCN of control group, structural changes like amputation neuroma and Wallerian degeneration were observed milder in the SCN of treatment group (Fig. 4A and B). In the SCN of control group, there is an intense inflammatory response caused by many granulocytes (Fig. 4F and I). Because anti-MT-I/II antibody (E-9) does not recognize rhMT-IIA, it is thought that the results of immunohistochemistry indicate the expression level of endogenous MT-I/II. The administration of rhMT-IIA significantly suppressed a decrease of endogenous MT in the ligation and distal site (Fig. 4C, D and K).

It has been reported that GAP43 plays an important role at growth cone of regenerating nerves and guides nerves to an appropriate direction thus providing as an index of neuronal regeneration[5]. On the other hand, glial fibrillary acidic protein (GFAP), an intermediate filament specific for glial cells including Schwann cells, is revealed that this protein has a critical role for maintenance for pain behaviors in addition to astrocyte activation[23]. Therefore, we next examined expression of GAP43 (Fig. 5A) and GFAP (Fig. 5B) in the SCN. In the SCN treated by PBS, there were few GAP43-positive nerve fibers (Fig. 5A-c) around 1 cm distal to the injury site. GAP43 also gradually decreased from the injury site to the more distal side (Fig. 5A-c and d). Even with PSL, expression of GAP43 was restored by administration of rhMT-IIA and intense staining was detected at more distal side (Fig. 5A-b), which seemed to be similar to that of SCN in healthy rat (Fig. 5A-e). Expression of GFAP was markedly up-regulated by PSL at both distal and proximal side of SCN (Fig. 5B-d, e, and f). In a clear contrast, intensity of GFAP significantly decreased by rhMT-IIA injection (Fig. 5B-a, b, and c). An apparent decrease of GFAP intensity is evident especially at the vicinity of the injury site (Fig.5B-b) with rhMT-IIA comparing to that with PBS.

4. Discussion

We previously reported that MT was expressed in Schwann cells and MT lacked in the injued peripheral nerves of CRPS-2 patients and painful neuroma[33]. In this report, we studied whether MT was related to neuropathic pain using PSL rats.

By immunohistochemistry, we observed that MT was

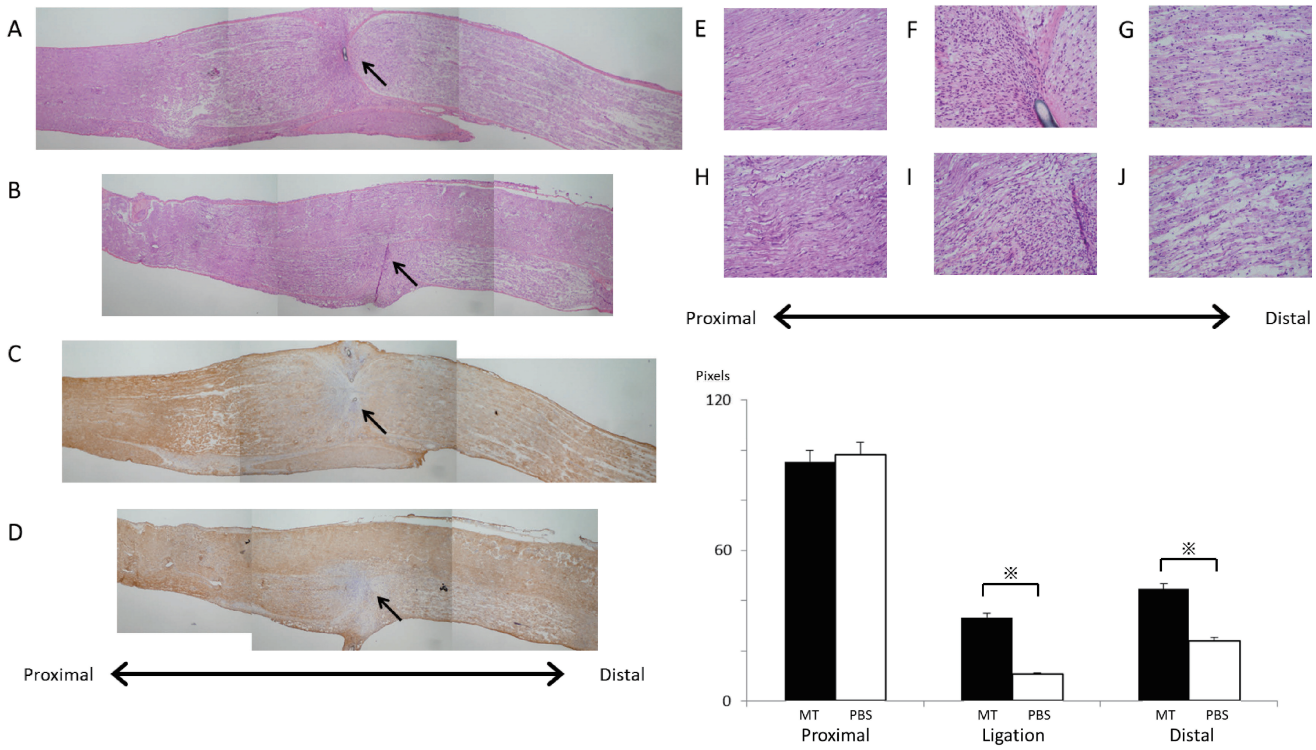

Fig. 4. Effect of recombinant MT on the expression level of endogenous MT. The rhMT-IIA protein (A, C, E, F, G) or PBS (B, D, H, I, J) was injected as described in "Materials and Methods". The injured nerve was resected at 7 days after ligation. Tissue sections were performed hematoxylin-eosin staining (A, B, E, F, G, H, I, J) and immunostaining with anti-MT (C, D). Sections of the sciatic nerve from PSL model rats were represented x40 (A, B, C, D) and x100 (E, F, G, H, I, J). Black arrows indicate the ligation point. Bar graph are shown as the mean ± SEM of 4 rats.

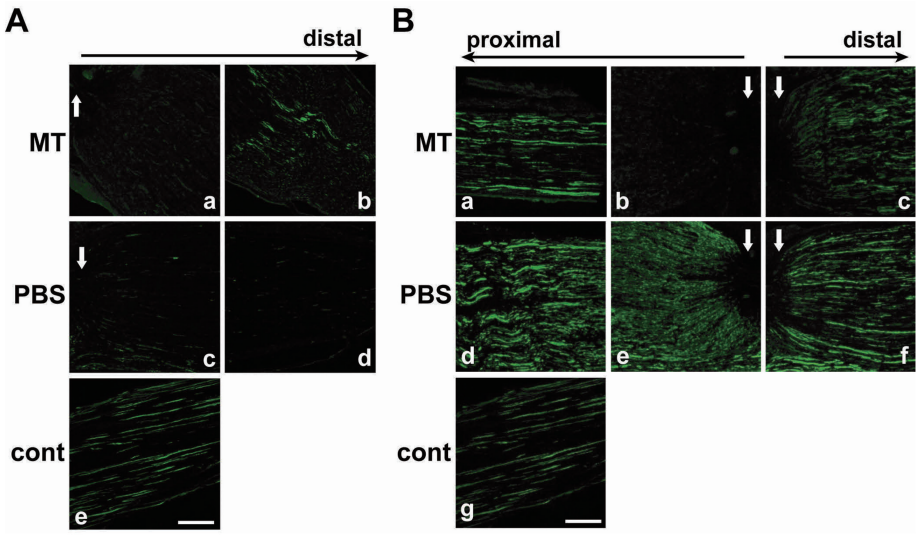

Fig. 5. Changes in GAP43 and GFAP expression in SCN of PSL rats. The rhMT-IIA protein (MT) or PBS (PBS) was injected as described in "Materials and Methods". Immunofluorescent staining of paraformaldehyde-perfused SCN was performed using antibodies against GAP43 (A) or GFAP (B) and Alexa Fluor 488-conjugated secondary antibodies. The results of immunofluorescent staining of SCN of non-PSL rats (normal) were shown in panel A-e and B-g. White arrows indicate the injury sites. Bar = 200 mm.

reduced in the distal and the proximal region of injury site at 28 days after PSL. We also confirmed that tactile allodynia and thermal hyperalgesia were occured after PSL. Recently, a correlation between ROS and neuropathic pain was noted. MT is oxidized by defensing against oxidative stress and scavenging for ROS. It is considered to cause a degradation of oxidized MT early[30]. The decrease of MT may participate in pain in PSL rats. Anti-MT-I/II monoclonal antibody does not recognize rhMT-IIA which has extra sequence at the N-terminus comparing with endogenous MT-I/II. In Fig. 4, therefore, it was detected only endogenous MT-I/II, not administered recombinant

protein. Administered rhMT-IIA was oxidized and degraded first, therefore a decrease of endogenous MT-I/II may be mainteined and suppress the pain behavior.

The MT promoter region has many response elements that regulate the transcription[14,15]. It has been reported that peripheral nerve injury induces the production of ROS and nitric oxide (NO) in axotomized neurons[9,13,24,46]. ROS may induce MT through multiple pathways, such as direct stimulation of an antioxidant response element and specific metal response elements (MREs) in the promoter region of MT gene[3,38]. The inhibit of NO production has been found to dampen the induction of MT by lipopolysaccharide in rat primary cultures[2]. This effect was also demonstrated in vivo, where NO suppression was shown to blunt stress-related MT-I upregulation in brain and liver of mouse[32], concerning a role of NO in MT induction. Transcriptional regulation of MT gene by metals is conferred by MRE in the MT promoter region[1,39]. The mRNA levels of MT in rats exhibit a hyperbolic response to increasing dietary zinc intake but little change in response to dietary copper7). This result agrees with the observed selectivity of zinc-induced MRE-binding transcription factor 1 binding to an MRE sequence[6]. Schwann cells are the main source of many inflammatory cytokines, such as tumor necrosis factor a and ILs, following a peripheral nerve injury28,43). Moreover, IL-6 was expressed in injured sciatic nerve[8]. IL-6 regulates the expression of MT by inducing tyrosine phosphorylation of signal transducers and activator proteins that interact with sites in the promoter region of the MT gene[27]. Glucocorticoids play a role in the IL-6-mediated induction of hepatic MT, and synergy between the two appears to require the physical interaction of their respective response elements[21,22]. As mentioned above, the expression of MT is regulated by various response elements. The administration of rhMT-IIA can reduce ROS level, NO and inflammatory cytokines, so that the consumption of endogenous MT may be suppressed .

There are few reports about the relation between neuropathic pain and metallothionein. In the present study, we showed that the decrease of MT in surrounding the ligation site may be strongly related to the initiation and/or the maintenance of neuropathic pain after the PSL. Moreover, the increase of endogenous MT by the administration of rhMT-IIA attenuated the painful behaviors. Lanza et al reported that mRNAs of MT-I / II were downregulated in the distal and proximal nerve portion after transections of median nerve of rats[26]. Ji et al. reported that MT 1a were up-regulated in dorsal root ganglion cells of the spinal nerve ligation model rats[19]. In contrast, Kwon et al reported that treatmrnt of MT-I siRNA atennuated painfull behaviors after chronic constriction injury of the rat sciatic nerve[25]. It has been reported that the administration of MT improved neurodegenerative disorders in the CNS[16,17,20,36,37]. The change in expression of MT in the pathogenesis of various diseases differs in diseases, such as neuropathic pain, Parkinson disease, Alzheimer disease and experimental autoimmune encephalomyelitis, and organs, such as brain, spinal cord and peripheral nerve. It is certain that there is strong relation between MT and neuropathic pain, however their details are still unknowm.

GAP43 plays a key role in guiding the growth of axons and is a major constituent of the growth cone that is upregulated in mammals during PNS regeneration[5]. In the distal region of ligation point, the expression level of GAP43 was upregulated in rhMT-IIA administered PSL rats. It is thought that rhMT-IIA promoted axon outgrowth during injured nerve regeneration. GFAP is an intermediate filament specific for glial cells including Schwann cells and is revealed that this protein has a critical role for maintenance for pain behaviors in addition to astrocyte activation[23]. After nerve damage, Schwann cells were activated and released proinflammatory cytokines which modulate spontaneous nociceptor activity and stimulus sensitivity. Injured Schwann cells also express GFAP. Administered and/or endogenous MT can suppresse Schwann cell activation, so that the expression of GFAP was reduced. However, the mechanisms of axon outgrowth by MT and suppression of Schwann cells activation by MT were not clearly revealed.

In conclusion, the expression level of MT reduced also in injured nerve of PSL rats and an administration of MT-I/II for the injury site of a peripheral nerve improves the painful behavior of the PSL rats.

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メタロチオネインは坐骨神経部分結紮モデルラットにおける神経障害性疼痛に寄与している

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神経障害性疼痛とは神経損傷もしくは神経疾患が原因となり，慢性疼痛を有する状態である．症状は自発痛，痛覚過敏，アロディニアを含む．神経障害性疼痛は中枢神経系と末梢神経系の両方の経路で発症する．さらに，神経細胞とグリア細胞の両方が神経障害性疼痛の発症に関与している．しかし，神経障害性疼痛の発病のメカニズムは明確に理解されていない．以前に私たちはプロテオミクスの手法を用いて，複合性局所疼痛症候群（CRPS）患者より採取した末梢神経ではメタロチオネインが欠損していることを報告した．本報告では，メタロチオネイン（MT）の濃度が，神経障害性疼痛モデルである部分的坐骨神経結紮（PSL）モデルラットにおいて変化したかどうかと，メタロチオネインの投与により，PSLモデルラットの物理刺激また温度刺激がもたらす行動に影響を及ぼすかどうかを検証した．MT-I/IIの発現は損傷部位の遠位側で徐々に減少を認めた．結紮後28日目では，MT-I/IIの発現は近位側と遠位側で同じ程度に著しく減少した．MTの投与はPBSを投与した群と比較し，アロディニアと温熱過敏の両方を有意に改善させた．さらに，神経再生のマーカータンパク質であるGAP43は遠位側で増加し，炎症のマーカータンパク質であるグリア線維性酸性タンパク質（GFAP）は損傷部位の近位側で減少した．これらの結果によりメタロチオネインは神経障害性疼痛の発症と，PSLラットにおける損傷された神経の再生に深く関与していると考えられる．