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Metallothionein deficiency in the injured peripheral nerves of complex regional pain syndrome as revealed by proteomics

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ABSTRACT

Complex regional pain syndrome (CRPS) is characterized by persistent and severe pain after trauma or surgery; however, its molecular mechanisms in the peripheral nervous system are poorly understood. Using proteomics, we investigated whether injured peripheral nerves of CRPS patients have altered protein profiles compared with control nerves. We obtained nerve samples from 3 patients with CRPS-2 who underwent resection of part of an injured peripheral nerve. Sural nerves from fresh cadavers with no history of trauma or neuropathic pain served as controls. Proteomic analysis showed that the number and functional distribution of proteins expressed in CRPS and control nerves was similar. Interestingly, metallothionein was absent in the injured nerves of CRPS-2, although it was readily detected in control nerves. Western blotting further confirmed the absence of metallothionein in CRPS-2 nerves, and immunohistochemistry corroborated the deficiency of metallothionein expression in injured nerves from 5 of 5 CRPS patients and 2 of 2 patients with painful neuromas. In contrast, all control nerves, including 5 sural nerves from fresh cadavers and 41 nerves obtained from surgically resected tumors, expressed MT. Furthermore, expression of S100 as a marker for Schwann cells, and neurofilament M as a marker of axons was comparable in both CRPS-2 and controls.

Metallothioneins are zinc-binding proteins that are probably involved in protection against injury and subsequent regeneration after CNS damage. Their absence from the injured peripheral nerves of patients with CRPS-2 suggests a potential pathogenic role in generating pain in the damaged peripheral nerves.
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1. Introduction

Complex regional pain syndrome (CRPS) is a chronic pain disorder distinguished by significant autonomic features that typically develop in an extremity after acute tissue trauma. In addition to neuropathic pain characteristics such as burning pain, hyperalgia, and allodynia, CRPS is associated with local edema and changes suggestive of autonomic involvement, such as trophic changes to the skin, hair, and nails, and altered motor function [44,45]. CRPS is subdivided into type 1 (CRPS-1) and type 2 (CRPS-2). CRPS-1 is frequently triggered by tissue injury but without underlying nerve injury. Patients with CRPS-2 experience similar symptoms which are clearly associated with nerve injury. However, there is no evidence that they differ in terms of pathophysiologic mechanisms or treatment responsiveness. CRPS is one of the most challenging chronic pain conditions to treat successfully. There is no definitive medical treatment, and clinical trials have failed to support the efficacy of many commonly used remedies. This lack of adequate treatment partly stems from incomplete understanding of its pathophysiologic mechanism(s).

The pathophysiologic mechanisms of CRPS seem to be multifactorial, and involve both peripheral and central nervous system mechanisms [35,38]. One suggestion implicates nociceptive neuropeptides released by injured primary afferent nerve fibers that increase firing in response to nociceptive stimuli and decrease the firing threshold for thermal and mechanical stimuli [20]. We previously reported that resection of injured peripheral nerves markedly relieves neuropathic pain in patients with CRPS-2 [48]. Histological examination of the resected nerves showed Wallerian

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degeneration, as well as up-regulation of substance P. These observations suggest that the injured nerve may express specific proteins such as neuropeptides or cytokines, resulting in pain and hyperalgesia by direct or indirect actions.

If proteins specifically expressed in the injured nerves contribute to the pathogenesis of CRPS, it might be possible to use proteomics to detect increased or decreased protein levels in the nerves. To test this hypothesis, we analyzed protein profiles in injured nerves resected from 3 patients with CRPS-2 using a proteomic approach. Sural nerves from fresh cadavers served as controls. The numbers of proteins detected and their functional distribution were similar in the injured and control nerves. Although no proteins were specifically up-regulated in CRPS nerves, we identified an absence of metallothionein in the injured nerves of CRPS, which was confirmed by Western blotting and immunohistochemistry.

MT are zinc-binding proteins that are possibly involved in metal homeostasis of free radical scavengers [1,19] and that may be important in protecting the CNS from injury and in its regeneration after injury as reported recently [5,6,12,28,29,37]. This lack of MT in injured peripheral nerves of patients with CRPS-2 suggests a potential pathogenic role of MT in pain generation in the injured peripheral nerves.

2. Methods

2.1. Nerve samples

The Sapporo Medical University ethics committee approved the study described in this report, and the 7 patients (2 males and 5 female; mean age 50 years; age range 35–76 years) with neuropathic pain who underwent resection of part of an injured peripheral nerve provided written informed consent. The mean period from the onset of CRPS to surgery was 18.1 months (range, 6–30 months). Five patients were diagnosed as having CRPS-2 and 2 patients as having painful neuroma. Diagnosis of CRPS was based on the IASP criteria [15]; in 5 patients it followed iatrogenic injury, and in 2 patients the pain developed after cuts. All patients had failed conservative treatment including medication, physiotherapy, and sympathetic ganglion block for at least 6 months. Five patients with CRPS-2 and 1 patient with painful neuroma had paresthesia and burning pain in the area innervated by the damaged nerve, and tenderness at the site of the injury. The remaining patient (case 7) had severe phantom pain caused by forearm amputation. Mean visual analog scale before surgery was 7.9 points (10 points maximum) as summarized in Table 1. Before surgery, we infiltrated lidocaine into the tender point for all patients and confirmed the disappearance or substantial attenuation of the symptoms. The nerve in the area in which the patient felt pain and a further 3 cm proximal to the site of injury was resected. Excised peripheral nerves from 3 patients (patients 1, 2, and 3) were snap-frozen in liquid nitrogen and kept at −80°C until proteomic analysis. The nerve specimens from all 5 patients were also fixed with formalin and stored for immunohistochemistry.

For controls, we excised sural nerves from fresh cadavers after obtaining written informed consent antemortem and written informed consent from the patient's family.

2.2. Sample preparation for sodium dodecyl sulfate–polyacrylamide gel electrophoresis

A 5-mm nerve segment was homogenized (ASONE homogenizer; Tokyo, Japan) in 200 μL of lysis buffer containing 40 mM Tris-base, 0.5% Triton X-100, 10% glycerol, 5 mM ethylenediaminetetraacetic acid (EDTA), and 2 mM Pefabloc(Roche, Indianapolis, IN);150 μL of the soluble fraction underwent trichloroacetic acid precipitation and was then dissolved in sodium dodecyl sulfate (SDS)–Urea buffer (0.2 M Tris–HCl, 4% SDS, 8 M urea, 0.1% dithiothreitol, and 0.01% bromophenol blue). With 200 μg protein of soluble or insoluble fraction, SDS–polyacrylamide gel electrophoresis was loaded on homemade 8% to 20% gradient polyacrylamide gels and electrophoresed under constant current of 40 mA per gel until the dye front reached 10 cm from the top of the gel. After electrophoresis, proteins were visualized with a silver-staining kit (EzStain; ATTO, Tokyo, Japan).

2.3. Mass spectrometry

Stained gels were cut into bands 5 mm in length based on the protein staining, and these bands were further divided into small cubes approximately 1 mm square as described elsewhere [49]. After reduction and alkylation, in-gel digestion was performed with trypsin (Roche) overnight at 37°C as described by Shevchenko et al. [39]. Peptides were subsequently separated on a HiQsilC18W-3 column (100 μm ID × 100 mm; KYA Technology, Tokyo, Japan). Elusion solvent A was 0.1% trifluoroacetic acid (TFA), whereas solvent B was 0.1% TFA in 70% ACN. The gradient was 5% to 50% for solvent B over 50 minutes at a flow rate of 300 nL/min. Separated peptides were spotted onto a 384-well AB OptiTOF MALDI Plate Inserts (AB Sciex, Foster City, CA). Peptide fractions were collected every 30 seconds, and the 150-nL peptide fractions were overlaid with 700 nLαCHCA (CHCA; Sigma, Tokyo, Japan) in 80 μg/mL ammonium citrate, 70% ACN, and 0.1% TFA. Mass spectrometric

Table 1
Case reports.

Case	Age/sex	Inciting event	Side	Area	Months[a]	Location	VAS		Isolated nerve	
							Preop	Postop		
1	38/F	Electric saw	L	Ulnar side of hand	11	Left hand	10	1	Dorsal branch of ulnar nerve	CRPS
2	47/M	Debridement	R	Thumb	15	Right thumb	8	2	First palmar digital nerve	CRPS
3	76/F	Osteosynthesis	R	Distal and radial forearm	16	Right distal forearm to hand	7	3	Palmar branch of median nerve	CRPS
4	44/F	Arthroplasty	R	Carpometacarpal area to distal radius	6	Right radial thumb	7	1	Dorsal branch of radial nerve	CRPS
5	56/F	Tenolysis	R	Thumb	30	Right thumb	9	0	First palmar digital nerve	CRPS
6	35/F	Knife	L	Radial side of hand	25	Right hand	8	0	Neuroma	Painful neuroma
7	54/M	Amputation	L	Forearm stump	24	Phantom pain	8	3	Neuroma	Painful neuroma

F = female; L = left; M = male; Postop = postoperative (1 year after nerve resection); Preop = preoperative; R = right; VAS = visual analog scale (for pain).
[a] Months denotes months elapsed until resection of painful nerve after initial cause.

analyses were performed on a 4800 Plus MALDI-TOF/TOF Analyzer (AB Sciex) with 4000 Series Explorer version 3.5 software (AB Sciex). Tryptic BSA standard (KYA Technology) and 6-peptide mixture (Absciex) was used to calibrate the mass accuracy. MS/MS acquisitions of MS spectra with S/N over 100 were carried out using air as the collision gas and a collision energy of 1 kV. Data were processed by Protein Pilot version 2.0 and version 3.0 (AB Sciex) using the Paragon search algorithm [40]. MS/MS data was searched against the human International Protein Index (IPI) database (version 3.63). The molecular function and biological role of proteins with more than 66% confidence was investigated using the Celera Discovery System as shown in Fig. 1 [21].

2.4. Western blotting analysis

A 5-mm-long nerve segment was homogenized in 100 μL T-PER Tissue Protein Extraction Reagent (Thermo, Yokohama, Japan) containing 1 μL Halt protease inhibitor (Thermo) and 1 μL of 0.5 M EDTA. Western blotting of MT was performed as described by Mizzen et al. with some minor modifications [26]. A 10-μg quantity of protein from each sample was electrophoresed on 10% to 20% SDS–PAGE (Bio-Rad, Tokyo, Japan) and using a transfer tank, transferred to a polyvinylidene fluoride membrane (Millipore, Tokyo, Japan) in transfer buffer (10 mM 3-cyclohexylamino-1-propanesulfonic acid containing 2 mM $CaCl_2$) at 40 V for 1 hour. The membrane was incubated in 2.5% glutaraldehyde for 1 hour and washed for 5 minutes 3 times in PBS. Mono-ethanolamine was added to 50 mM for the third wash. The membrane was blocked in 5% skim milk and incubated with antibodies against MT (1:100; code M0639, lot 10036820; DAKO, Tokyo, Japan), and actin (1: 500; product no. A2103, lot 087k4841; Sigma, Tokyo, Japan,) for 2 hours. Immunoreactive bands were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo).

2.5. Immunohistochemistry

The nerve segments were fixed in 10% formalin and paraffin embedded. Sections cut at 3 μm were deparaffinized and treated with 3% hydrogen peroxide for 10 min, and then washed in PBS. The sections were incubated in pH 9 buffer (code 415211; Nichirei Biosciences, Tokyo, Japan) at 105°C for 15 minutes for antigen retrieval. The sections were then incubated with anti-MT (1:50; code M0639, lot 10036820; Dako), anti-S100 (100:1; code N1573, lot 10035610A; DAKO), and anti-neurofilament M (1:100; catalog

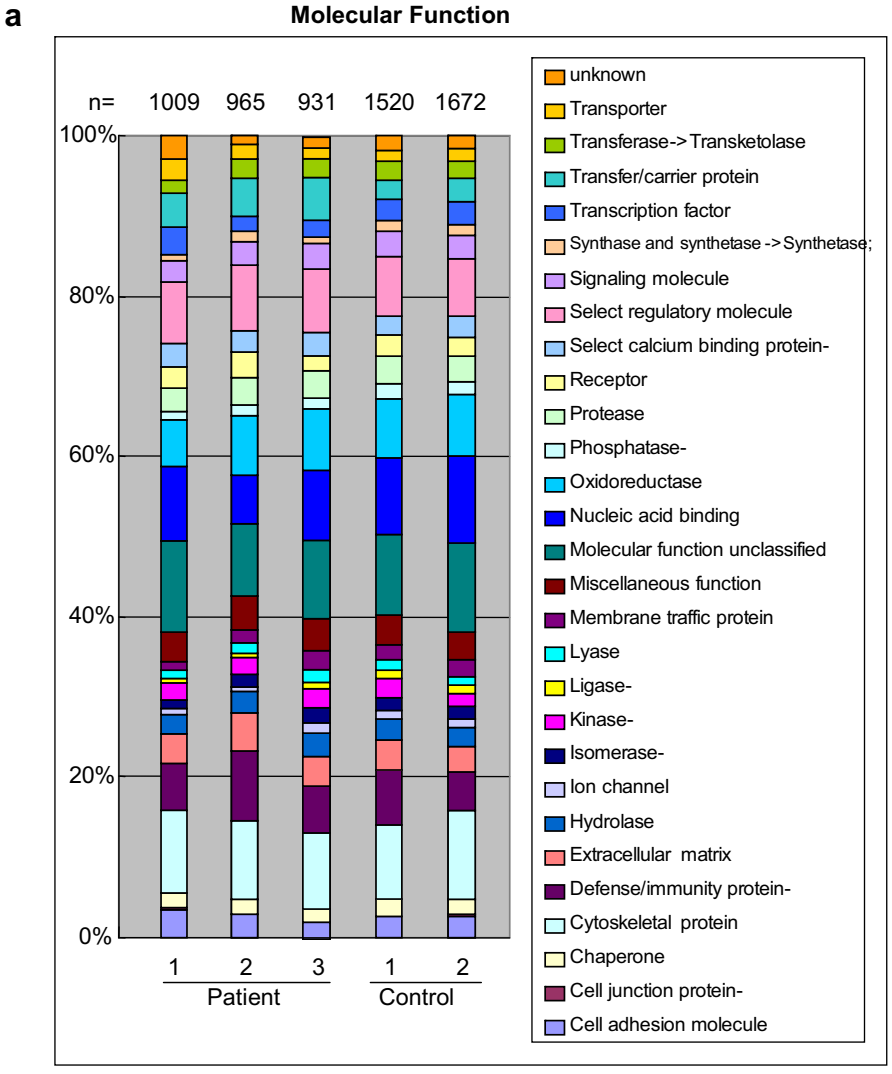

Fig. 1. Molecular function and biological process of assigned proteins in nerves of CRPS and controls. (a) Proteins assigned were analyzed according to their possible role in molecular function using the Celera Discovery System (CDS). The ratio of molecules for each function is shown for patients 1, 2, and 3 and for controls 1 and 2. No obvious differences were observed. (b) Proteins assigned were analyzed for their possible role for biological process using CDS. The ratio of molecules for each process is shown for patients 1, 2, and 3 and for controls 1 and 2. No obvious differences were observed.

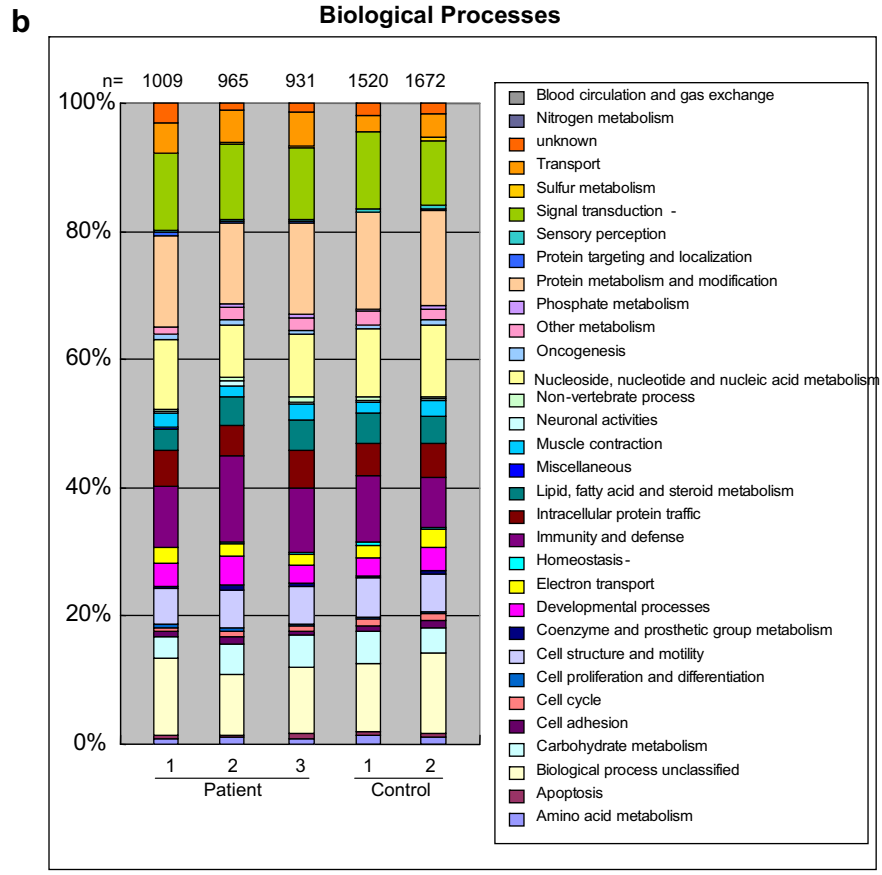

Fig. 1 (*continued*)

no. 13-0700, lot 1402037; Invitrogen, Tokyo, Japan) for 1 hour at room temperature. The sections were further incubated with the corresponding secondary antibody and developed with diaminobenzine (DAB; DAKO).

3. Results

3.1. Comparable proteome in CRPS and control nerves

We used proteomics to analyze 3 CRPS nerves and 2 control nerves obtained from fresh cadavers. All of the proteins assigned in this study are listed in Supplementary Table S1. We compared the protein profile of each nerve sample and classified their molecular functions. There were no obvious changes of constitution between CRPS and control nerves (Fig. 1). The majority of proteins detected were present in both the CRPS group and controls.

3.2. Lack of MT in CRPS nerves at the peptide level

Table 2 shows proteins not detected in the CRPS nerves but readily found in the control nerves by mass spectrometric analysis with the number of peptides for protein assignment. The number

Table 2
Peptide numbers detected in each sample for corresponding proteins.

Protein name	Accession No.[b]	Number of peptides observed[a]				
		CRPS			Cadaver	
		1	2	3	1	2
Metallothionein 2A	spt|P02795	0	0	0	8	8
Metallothionein-1H	spt|P80294	0	0	0	1	9
C-reactive protein	spt|P02741	0	0	0	1	3
OTTHUMP00000030191	trm|Q9NTT1	0	0	0	1	1
PHYHD1 protein	trm|Q7Z7P9	0	0	0	1	1
Metallothionein-2	spt|P80295	0	0	0	4	5
Metallothionein-1G	spt|P13640	0	0	0	4	4
Metallothionein	trm|Q86YX3	0	0	0	5	3
S100 A4[c]	cra|hcp33585.3	17	14	19	17	29
Neurofilament M	spt|P07197	591	517	590	912	934

[a] Peptides observed in each sample over 95% of probability with PARAGON algorithm are depicted. Number indicates number of peptides detected and suggested the presence of the proteins indicated as described in the results.
[b] Accession No. is based on the CDS (Celera Discovery System; Celera, Alameda, CA). spt, trm, and cra are names in protein database. Spt, SWISS-PROT; trm, TrEMBL.
[c] Peptides derived from S-100A4 and Neurofilament M are similarly detected in CRPS and cadaver samples.

of peptides is correlated with the amount of corresponding proteins under defined conditions as described [25,41]. The number of peptides with high confidence by mass spectrometry provides qualitative information about the amount of corresponding proteins. Therefore, quantitative validation of protein levels is essential. Peptides derived from MT were abundant in the control nerves with a high probability. In marked contrast, no MT peptides were detected in any of the CRPS samples. Although C-reactive protein precursor, OTTHUMP00000030191, and PHYHD1 proteins were also detected only in the controls and not in the CRPS samples, the peptide number of MT was far greater than for these proteins, indicating apparent changes of MT in the CRPS nerves. The number of peptides derived from S-100A4 and Neurofilament M are also shown in Table 2, and were found to be similar in CRPS and cadaver samples. We therefore decided to expand our studies of MT further at the protein level.

3.3. Lack of MT in CRPS nerves by Western blotting and immunohistochemistry

Western blotting showed an apparent expression of MT in the nerves from fresh cadavers but not in the CRPS samples (Fig. 2a). MT expression was barely detectable in all of the CRPS samples, whereas intense signals were detected with 3 of 3 control samples.

Fig. 2b shows the immunohistochemistry results for MT, S100, and neurofilament M (NFM) in the CRPS and control nerves, this procedure confirms the lack of MT, although apparent staining of S100 and NFM was detected in this CRPS nerve. We chose S100 and NFM protein expression as good indicators for Schwann's cells and neurofilament M for axon, respectively. Lack of MT seems to be nerve specific, as endothelial cells surrounding these nerves exhibited positive staining (data not shown). Importantly, S100 and NFM

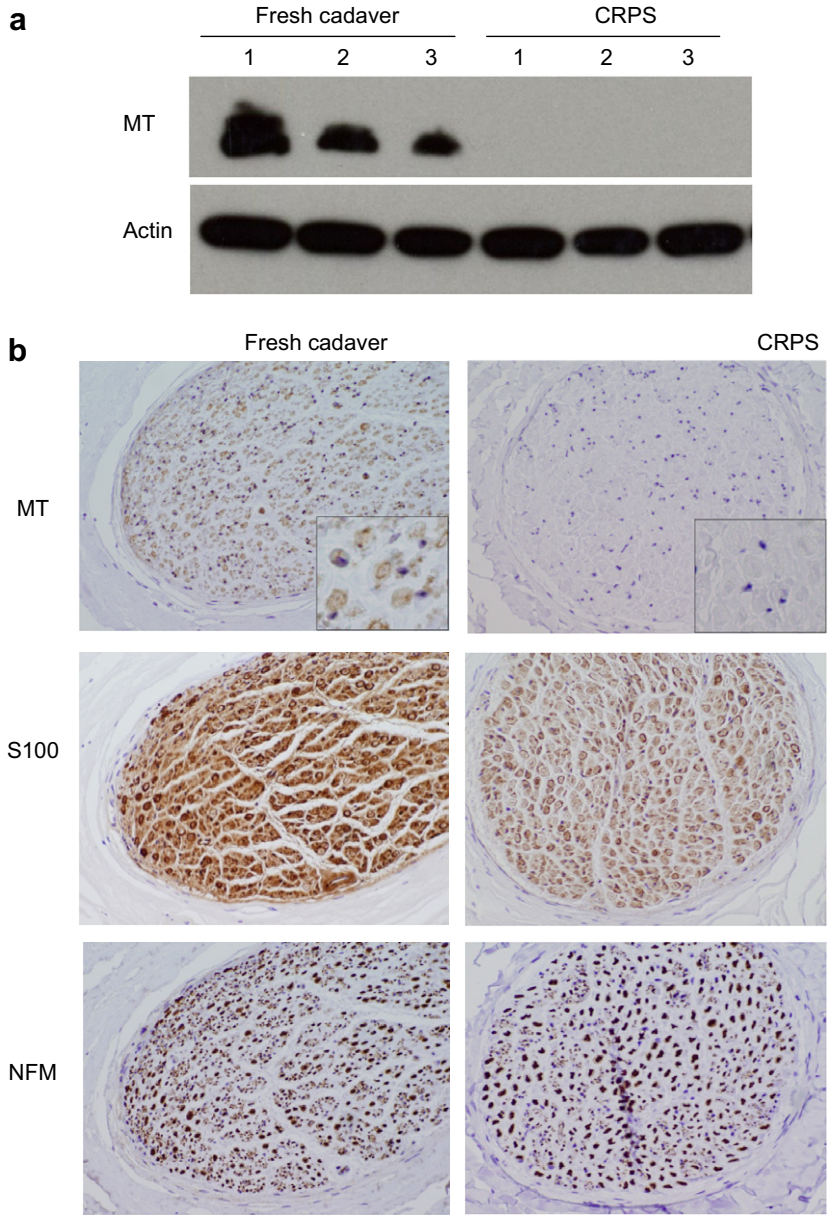

Fig. 2. Metallothionein was detected in control but not CRPS nerves by immunoreactivity. (a) Western blotting of metallothionein (MT) in the nerves of CRPS and control nerves obtained from fresh cadaver. The intense band of MT is readily detected in all samples obtained from fresh cadavers 1, 2, and 3, respectively, but not in those of CRPS 1, 2, and 3, respectively. Actin is ubiquitously observed irrespective of samples, depicting that the loading amount of tissue extracts is similar. (b) Nerve-related proteins, including S100 and neurofilament M (NFM), are equally expressed in nerves of CRPS and fresh cadaver, with a relative decrease in S100 staining in CRPS nerve. MT is totally absent in the nerve of CRPS, although apparent staining is observed in that of fresh cadaver. Original magnification is 100×. Insets represent higher magnification (400×).

Table 3
Expression of MT, S100 and NFM in the affected nerves of CRPS and sural nerve of fresh cadavers.

	MT	S100	NFM
CRPS 1	–	+	+
CRPS 2	–	+	+
CRPS 3	–	+	+
Fresh cadaver 1	++	++	+
Fresh cadaver 2	+	++	+
Fresh cadaver 3	++	++	+

NFM = neurofilament M; S100 = S100 protein; – = no reaction; + = moderate reaction; ++ = strong reaction.

Table 4
Expression of MT in the nerves derived from various samples.

	N	Age (y)	M:F	Positive[a]	Negative[b]
CRPS	7	50 ± 14	2:5	0	7
Fresh cadaver	5	84 ± 7.1	3:2	5	0
Benign tumor	5	51 ± 16.4	2:3	5	0
Malignant tumor					
Induction chemotherapy+	10	50.2 ± 15	4:6	0	0
Induction chemotherapy–	26	64.7 ± 16.9	12:14	26	0

F = female; M = male.
[a] Number of samples expressing MT.
[b] Number of samples lacking MT expression.

were expressed in both control and CRPS samples, indicating preserved nerve structures in both types of nerves. Expression of MT, S100 and NFM in the 3 CRPS and 3 cadaver nerves is summarized in Table 3.

3.4. Immunohistochemistry further confirmed lack of MT in CRPS nerves

Studies using Western blotting and immunohistochemistry confirmed lack of MT in CRPS nerves, although postmortem changes might explain the robust expression of MT in the control nerves. To exclude this possibility, we examined the expression of MT in peripheral nerves taken from histopathological specimens of 41 patients with either benign or malignant bone and soft tissue tumors who had undergone wide excision. As shown in Fig. 3, all of the nerves involved in tumors clearly expressed MT. In contrast, no MT signal was detected in 5 injured nerves of CRPS and 2 painful neuromas (Table 4).

4. Discussion

In recent years, numerous studies based on proteomics have been published. Significant results were obtained in diverse fields such as malignant tumors [13,18], blood diseases [2,17], and Alzheimer's disease [36,47]. Proteomics offer new possibilities in studies of changes in protein expression involving specific organs directly and inclusively. We here describe our use of proteomics to compare detailed protein profiles in the injured nerves of CRPS patients with that of control nerves obtained from human cadavers. We found a lack of MT in CRPS nerves which was subsequently confirmed by Western blotting and immunohistochemistry. We chose S100 and NFM as a positive control of IHC because these 2 proteins should be expressed both in control and injured peripheral nerves. S100 is normally present in cells derived from the neural crest such as Schwann cells, melanocytes, and glial cells, and has various intracellular and extracellular functions such as

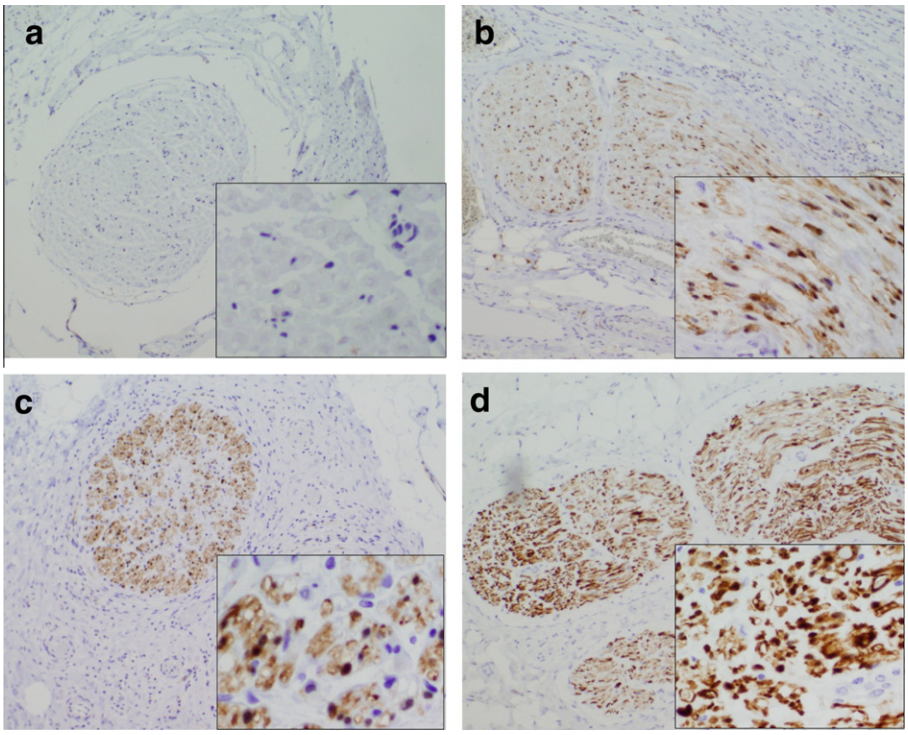

Fig. 3. Expression of MT in nerves involved in surgically resected tumors but not in painful neuroma. Peripheral nerves involved in surgically resected tumors are stained for MT to examine expression of MT. All of the nerves summarized in Table 4 showed apparent expression of MT except the CRPS nerves. (a) CRPS (case 6 in Table 1); (b) abdominal desmoid; (c) myxofibrosarcoma of left shoulder without induction chemotherapy; and (d) metastatic osteosarcoma of right rib with induction chemotherapy. Original magnification 100×. Insets represent higher magnification (400×).

regulating protein phosphorylation, transcription factors, and calcium ion homeostasis [7]. NFM is an intermediate filament of approximately 145 to 160 kDa and is expressed mainly in axons [14]. The results of immunohistochemistry suggest that MT is expressed in Schwann cells. We speculate that this could imply that MT deficiency in the injured peripheral nerves may underlie the development and/or continued generation of CRPS pain.

MTs are low-molecular-weight proteins (6–7 kDa) with abundant cysteine residues (close to 30% of all amino acid components) that 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 [3]. MTs are multifunctional proteins induced by various chemicals and cytokines such as glucocorticoids, interleukin (IL)-1, IL-6, tumor necrosis factor-α and reactive oxygen species (ROS) [28]. Their major functions are thought to involve maintaining homeostasis of intravital heavy metals, defense against oxidative stress and scavenging for ROS. In mammals, MTs are divided into 4 subfamilies (MT-1 to MT-4). MT-1 and MT-2 are widely expressed and regulated coordinately, whereas MT-3 and MT-4 are expressed predominantly in the central nervous system (CNS) and squamous epithelia, respectively [5]. The MT-1 and MT-2 isoforms are remarkably similar, so they are often discussed as a single isoform (MT-1/2) [11].

MT scavenges harmful substances involved in the pathophysiology of neurodegenerative conditions [8,9,24]. Mice with a null mutation of MT (MTKO) and brain injury caused by an occluded cerebral artery display increased inflammation, oxidative stress and apoptosis, and delayed recovery compared with those in wild-type mice [30]. Likewise MTKO, mice fared worse after stroke [46]. In contrast, MT-1 overexpression downregulates the inflammatory response, decreases oxidative stress and apoptosis, and promotes tissue repair after focal brain injury [29]. MT-1/2 was upregulated to protect the brain from oxidative stress in neurodegenerative diseases such as Alzheimer's disease [16], experimental autoimmune encephalomyelitis [31] and amyotrophic lateral sclerosis [34]. Other studies have indicated that administration of MT-1/2 improved neurodegenerative disorders of the CNS [32,33].

In contrast, there are few reports concerning MT in normal and pathologic peripheral nerve conditions [42,43]. The caliber of the large myelinated fibers of phrenic nerves in MT-1/2 knockout mice (MTKO) was reduced and MT-3 was inhibited in peripheral nerve regeneration. Regeneration of peripheral nerves was inhibited in MT-1/2 knockout mice and enhanced in MT-3 knockout mice, suggesting that MT-1/2 promotes peripheral nerve regeneration. We performed a comprehensive analysis of the protein profiles of the injured nerves of patients with CRPS-2 by using a proteomic approach, and demonstrated a lack of MT in the nerves. MT deficiency in the injured peripheral nerve may inhibit its regeneration, leading to development of CRPS.

Both peripheral and central nervous system mechanisms are involved in CRPS [4,35,38]. These include peripheral and central sensitization, inflammation, altered sympathetic and catecholaminergic function, altered somatosensory representation in the brain, genetic factors, and psychophysiological interactions. After nerve injury, the primary afferent fibers release several proprioceptive neuropeptides, such as substance P and bradykinin, that increase background firing of nociceptors, increase firing in nociceptive stimuli, and decrease the firing threshold for thermal and mechanical stimuli [20]. The latter 2 effects contribute, respectively, to the hyperalgesia and allodynia that are key diagnostic features of CRPS. Probably peripheral sensitization triggered by the initial tissue trauma develops early in CRPS patients. An increase in neuropeptide release can be regarded as physiological, whereas patients who develop CRPS may have a delayed resolution of the inflammatory cascade. Recently, a correlation between ROS and neuropathic pain was noted [10,22,23,27]. Peripheral ROS causes peripheral sensitization after central sensitization and persistent pain [4]. Because ROS are one of the major stimulants raising MT expression, and because MT subsequently scavenge ROS, disappearance of MT in the affected nerves significantly disrupts this protective machinery. We suggest that lack of MT may compromise protection and regeneration of the injured nerve, leading to disease progression.

Our study has several limitations. First, the time to lack of MT expression after trauma in the injured nerve is unknown. Also unknown is whether the lack of MT is limited to the injured area or is more widespread. In the clinical setting, all of the nerves are resected in part from patients with chronic stage of CRPS. An animal model for neuropathy would help to resolve these problems, and such a study is now progressing. Second, our patients were inhomogeneous in type of injury and disease duration and few in number. However, there was no exception to the finding of MT lack, but larger numbers might reveal cases of CRPS that express MT. Study of more CRPS nerves would resolve this question and give further insight on this pathological condition. Third, it is possible that MT deficiency is related exclusively to degeneration of peripheral nerves but not to pain generation. For obvious ethical reasons, we could not resect nonpainful nerves.

In conclusion, proteomic analysis is a promising new approach to studies of protein expression. Our study adds to the growing evidence of dysregulated oxidative stress and inflammatory responses in patients with CRPS and painful neuroma. These findings may have important implications for the treatment of these patients.

Conflict of interest

None of the authors has a conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pain.2011.11.008.

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