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Toxicology

Involvement of high mobility group box 1 in the development and maintenance of chemotherapy-induced peripheral neuropathy in rats

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Abbreviations: APC, activated protein C; CBP, cyclic-AMP regulatory element binding protein (CREB)-binding protein; DIC, disseminated intravascular coagulation; DRG, dorsal root ganglia; GAPDH, glyceraldehyde-3-phosphoate dehydrogenase; HAT, histone acetyltransferase; HDAC, histone deacetylase; HMGB1, high mobility group box 1; i.pl., intraplantar; LPS-RS, lipopolysaccharide from *Rhodobacter sphaeroides*; LMWH, low molecular weight heparin; PCAF, p300/CBP-associated factor; RAGE, receptor for advanced glycation endproducts; rhsTM, recombinant human soluble thrombomodulin; TLR4, Toll-like receptor 4

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Abstract

Given that high mobility group box 1 (HMGB1), a nuclear protein, once released to the extracellular space, promotes nociception, we asked if inactivation of HMGB1 prevents or reverses chemotherapy-induced painful neuropathy in rats and also examined possible involvement of Toll-like receptor 4 (TLR4) and the receptor for advanced glycation endproduct (RAGE), known as targets for HMGB1. Painful neuropathy was produced by repeated i.p. administration of paclitaxel or vincristine in rats. Nociceptive threshold was determined by the paw pressure method and/or von Frey test in the hindpaw. Tissue protein levels were determined by immunoblotting. Repeated i.p. administration of the anti-HMGB1-neutralizing antibody or recombinant human soluble thrombomodulin (rhsTM), known to inactivate HMGB1, prevented the development of hyperalgesia and/or allodynia induced by paclitaxel or vincristine in rats. A single i.p. or intraplantar (i.pl.) administration of the antibody or rhsTM reversed the chemotherapy-induced neuropathy. A single i.pl. administration of a TLR4 antagonist or low molecular weight heparin, known to inhibit RAGE, attenuated the hyperalgesia caused by i.pl. HMGB1 and also the chemotherapy-induced painful neuropathy. Paclitaxel or vincristine treatment significantly decreased protein levels of HMGB1 in the dorsal root ganglia, but not sciatic nerves. HMGB1 thus participates in both development and maintenance of chemotherapy-induced painful

neuropathy, in part through RAGE and TLR4. HMGB1 inactivation is considered useful to prevent and treat the chemotherapy-induced painful neuropathy.

Keywords: high mobility group box 1; chemotherapy-induced painful neuropathy; neuropathic pain; thrombomodulin

1. Introduction

High mobility group box 1 (HMGB1), a nuclear DNA-binding protein, regulates transcriptional activities as a structural co-factor in mammalian cells. HMGB1, once released passively from dying cells, plays pro-inflammatory roles as one of damage-associated molecular patterns (DAMPs), while it is also actively secreted by certain cells including macrophages (Malarkey and Churchill 2012; Yanai et al. 2012). The nuclear HMGB1 can be acetylated by histone acetyltransferases (HATs) such as, cyclic-AMP regulatory element binding protein (CREB)-binding protein (CBP) and p300/CBP-associated factor (PCAF) (Ong et al. 2012; Wu et al. 2012), and deacetylated by histone deacetylases (HDACs), particularly HDAC1 and HDAC4 (Evankovich et al. 2010). The acetylated HMGB1 is translocated to the cytoplasm, and then released to the extracellular space following its packaging into secretory lysosomes that is regulated by calcium/calmodulin-dependent protein kinase (Zhang et al. 2011). Accumulating evidence strongly suggests that HMGB1 plays a pronociceptive role in processing somatic and visceral pain (Agalave and Svensson 2014; Tanaka et al. 2013; Tanaka et al. 2014). We have demonstrated the pronociceptive role of peripheral HMGB1 in rodents with inflammatory hyperalgesia induced by intraplantar administration of lipopolysaccharide (Tanaka et al. 2013) and with bladder pain accompanying cyclophosphamide-induced cystitis (Tanaka et al. 2014). The pronociceptive

roles of HMGB1 in the spinal cord and/or dorsal root ganglia (DRG) have been well described in the inflammatory pain mouse model with the collagen antibody-induced arthritis (Agalave et al. 2014) and in the rodents with the neuropathy induced by surgical nerve injury (Allette et al. 2014; Feldman et al. 2012; He et al. 2013; Kuang et al. 2012; Nakamura et al. 2013; Otoshi et al. 2011; Shibasaki et al. 2010) or by genetic type 2 diabetes (Ren et al. 2012). Nonetheless, it remains unclear whether HMGB1 participates in chemotherapy-induced neuropathic pain which could limit the life-saving cancer therapy.

Thrombomodulin, expressed on vascular endothelial cells, is a membrane protein which is composed of five domains: the N-terminal lectin-like domain [TM domain (TM-D) 1], EGF like domain (TM-D2), O-glycosylation-rich domain (TM-D3), trans-membrane domain (TM-D4) and C-terminal cytoplasmic domain (TM-D5) (Conway 2012). TM-D2 directly or indirectly inhibits thrombin-dependent coagulation by binding to thrombin or promoting the formation of activated protein C (APC). On the other hand, TM-D1 binds to HMGB1 and facilitates its degradation by thrombin. Recombinant human soluble thrombomdulin (rhsTM) that lacks TM-D4 and TM-D5 is used for the clinical treatment of disseminated intravascular coagulation (DIC) in Japan. Our previous study has shown that rhsTM prevents inflammatory pain and bladder pain and is available as a novel analgesic for treatment of HMGB1-mediated pain (Tanaka et al. 2013; Tanaka et al. 2014).

In the present study, to inactivate endogenous extracellular HMGB1, we used the rat

anti-HMGB1-neutralizing monoclonal antibody (Liu et al. 2007) and rhsTM that blocks HMGB1-dependent pain by sequestering HMGB1 and promoting it degradation by thrombin (Ito et al. 2008; Tanaka et al. 2013; Tanaka et al. 2014), and evaluated their preventive and/or therapeutic potentials in rats with the neuropathy induced by paclitaxel or vincristine, anti-cancer agents.

2. Materials and methods

2.1. Animals

Male Wistar rats (7-10 weeks old) were obtained from Kiwa laboratory Animals Co., Ltd. (Wakayama, Japan). Three to five rats were group-housed in each cage, maintained under a 12-h light/dark cycle and allowed access to water and food *ad libitum.* All animals were used with approval by Kindai (formerly Kinki) University's Committee for the Care and Use of Laboratory Animals, and all procedures were in accordance with the Guiding Principles approved by The Japanese Pharmacological Society and with the guidelines for animal experimentation of the International Association for the Study of Pain.

2.2. Major chemicals

The rat anti-HMGB1-neutralizing monoclonal antibody and control rat IgG were made in house, the specificity of the antibody being described elsewhere (Liu et al. 2007). The chicken anti-HMGB1-neutralizing polyclonal antibody, the control chicken IgY and HMGB1 from bovine thymus were purchased from SHINO-TEST Corporation (Kanagawa, Japan). Recombinant human soluble thrombomodulin (rhsTM) was provided by Asahi Kasei Pharma

(Tokyo, Japan). Lipopolysaccharide from *Rhodobacter sphaeroides* (LPS-RS) was obtained from InvivoGen (San Diego, CA, USA). Low molecular weight heparin (LMWH; molecular weight, 4500-6500; 79.5 U/mg), known to inhibit receptor for advanced glycation endproducts (RAGE) (Liu et al. 2009; Myint et al. 2006; Takeuchi et al. 2013), was a gift from Fuso Pharmaceutical Industries, Ltd. (Osaka, Japan). The rat anti-HMGB1 monoclonal antibody and rat IgG were dissolved in 0.01 M PBS. The chicken anti-HMGB1 polyclonal antibody and chicken IgY were dissolved in 0.2 M PBS. The rhsTM was dissolved in 0.002% Tween 80-containing saline. HMGB1, LPS-RS or LMWH was dissolved in saline.

2.3. Assessment of nociceptive threshold and paw swelling in rats

Nociceptive threshold was measured by the paw pressure test using an analgesia meter (MK-300, Muromachi Kikai Co., Tokyo, Japan) or von Frey test according to the up-down method. In the paw pressure test, pressure was applied to the hindpaw of rats at a linearly increasing rate of 30 g/s. The paw withdrawal threshold was determined and expressed as the percentage of the baseline value, and a cut-off value of 500 g was used to avoid damage to the paw (Kawabata et al. 2007). In the von Frey test, rats were placed in a plastic cage (34.2×29.4×17.8 cm) with a wire mesh bottom. After 1-h acclimatization in the cage, the hindpaw was stimulated with one of 8 distinct von Frey filaments with strengths of 2, 4, 6, 8,

10, 15, 26 and 60 g for 6 s and the 50% nociceptive threshold was determined as reported elsewhere (Chaplan et al. 1994). After the baseline threshold was determined 2-3 times daily for 3-5 days, the animals were randomly allocated to test groups without considering the responses to the chemotherapeutic drugs. The paw swelling was assessed by measuring the paw thickness using a tissue caliper with 0.05 mm accuracy, as described previously (Tanaka et al. 2013).

2.4. Creation of chemotherapy-induced neuropathic pain models in rats

A chemotherapy-induced neuropathic pain model was created as described previously (Polomano et al. 2001). After measurements of baseline nociceptive thresholds, rats were administered i.p. paclitaxel or vincristine according to the following schedules. Paclitaxel at 2 mg/kg [prepared with saline from 6 mg/mL solution in 50% Cremophor EL® (polyethoxylated castor oil) and 50% ethanol, Taxol® , Bristol-Myers Squibb, Co. Ltd., Tokyo, Japan] was repeatedly administered i.p. every two days (day 0, 2, 4 and 6), 4 times in all. Vincristine at 0.1 mg/kg (prepared in saline, Wako Pure Chem., Osaka, Japan) was administered i.p. daily in two 5-day cycles with 2-day break between cycles (day 0-4 and 7-11), 10 times in total (Flatters and Bennett 2004; Weng et al. 2003).

2.5. Drug administration schedules

For the assessment of their preventive effects on the chemotherapy-induced neuropathy, the rat anti-HMGB1 antibody at 1 mg/kg or rhsTM at 1-10 mg/kg was repeatedly administered i.p. to rats once a day for 7 days during paclitaxel treatment or for two 5-day cycles of vincristine treatment. Some rats received daily intraplantar (i.pl.) administration of the rat anti-HMGB1 antibody at 50 μg/paw in a volume of 100 μl, according to the same schedules during paclitaxel treatment. To assess their therapeutic effects on the chemotherapy-induced neuropathy, the rat anti-HMGB1 antibody at 1 mg/kg or rhsTM at 10 mg/kg was administered i.p. 14 days or more after the onset of paclitaxel or vincristine treatment. In some experiments, rats received i.pl. administration of the rat or chicken anti-HMGB1-neutralizing antibody at 50 μg/paw, LPS-RS at 50 μg/paw or LMWH at 10 μg/paw 14 days or more after the onset of paclitaxel or vincristine treatment. In separate experiments, the rats received i.pl. administration of LPS-RS at 50 μg/paw or LMWH at 10 μg/paw in a volume of 20 μl, 30 min before i.pl. administration of HMGB1 in a volume of 100 μl.

2.6. Determination of protein levels in the plasma and tissues

Under anaesthesia with i.p. administration of urethane at 1.5 g/kg, the blood was collected from the abdominal aorta into a syringe containing a one-tenth volume of 3.8% sodium citrate (Fuso Pharmaceutical Industries, Ltd.) 14 days or more after the onset of paclitaxel or vincristine. After the blood collection, the rats were transcardially perfused with 200 mL of saline, and the DRG at L4-L6 levels, plantar tissue of the hindpaw and sciatic nerves were excised. Plasma HMGB1 levels were determined using an ELISA kit for HMGB1 (SHINO-TEST Corporation). Tissue HMGB1 levels were assessed by Western blotting. Briefly, each tissue sample was homogenized and sonicated in a RIPA buffer containing PBS, 1% Igepal Ca-630 (Sigma-Aldrich, St. Louis, MO, USA), 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/mL phenylmethylsulfonyl fuluoride, 0.15 U/mL aprotinin and 1 mM sodium orthovanadate. After centrifugation, the supernatant was added the same volume of 2× electrophoresis sample buffer containing 19% glycerol, 5.7% SDS and 240 mM Tris-HCl (pH 6.7). After addition of 2-mercaptoethanol and bromo phenol blue, proteins in the sample were denatured at 95-100ºC for 5 min, separated by electrophoresis on 7.5% or 12.5% SDS-polyacrylamide gels (Wako Pure Chem., Osaka, Japan) for detection of HDAC1, HDAC4, HMGB1, TLR4, RAGE or glycelaldehyde-3-phosphate dehydrogenase (GAPDH), and transferred to the PVDF membrane (Immobilon-P, Merck Milipore Corporation, Billerica, MA, USA). The membrane was blocked with a blocking solution containing 5% skim milk, 137 mM NaCl, 0.1% Tween 20 and 20 mM Tris-HCl (pH 7.6). After washing, the membrane

was incubated with the affinity-purified anti-HMGB1 rabbit polyclonal antibody (1:40000 dilution, SHINO-TEST Corporation), the anti-HDAC1 mouse monoclonal antibody (1:3000 dilution, Cell Signaling Technology, Beverly, MA, USA), the anti-HDAC4 mouse monoclonal antibody (1:5000 dilution, Cell Signaling Technology), the anti-TLR4 rabbit polyclonal antibody (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), the anti-RAGE rabbit polyclonal antibody (1:1000 dilution, Abcam, Cambridge, UK) or the anti-GAPDH rabbit polyclonal antibody (1:3000 dilution, Santa Cruz Biotechnology) overnight at 4 ºC. After washing, the membrane was incubated with a secondary antibody, the HRP-conjugated anti-rabbit IgG (Merck Milipore) or anti-mouse IgG (Cell Signaling Technology), for 1.5 h. Immunopositive bands for HMGB1, HDAC1, HDAC4 and GAPDH around 29, 62, 140 and 37 kDa, respectively, were visualized by the enhanced chemiluminescence detection regent (Nacalai Tesque, Inc., Kyoto, Japan) and detected by Image Quant 400 (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The detected bands were quantified using a densitometric software (Image J downloaded from http://imagej.nih.gov/ij/download.html).

2.7. Immunohistochemical localization of HMGB1, TLR4 and RAGE in the sciatic nerves of rats.

Expression of HMGB1, TLR4 and RAGE in the sciatic nerves was examined by

immunohistochemistry 14 days after the onset of repetitive i.p. administration of paclitaxel. Under urethane (1.5 g/kg, i.p.), rats were transcardially perfused with 200 ml of saline. The sciatic nerves were removed and fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) for 3 days at 4°C, and then cryoprotected in 30% sucrose in 0.1 M PB at 4°C. The tissue was embedded in Cryomatrix™ (Thermo Fisher Scientific, Yokohama, Japan) and frozen. Sections (8 μm thickness) were cut on a cryostat and mounted onto hydrophilic silanized slides (Dako, Tokyo, Japan). The sections were dried at 37°C overnight, and then washed with phosphate-buffered saline (PBS) and incubated for 2 h in a blocking solution of 4% bovine serum albumin (BSA) in PBS with 0.1% Triton X-100 at room temperature. After blocking, the sections were incubated overnight at 4°C with a chicken anti-HMGB1 polyclonal antibody (#326059669, SHINO-TEST Corporation), a mouse anti-neurofilament 200 (NF200) monoclonal antibody (N0142, Sigma-Aldrich), a mouse anti-S100β monoclonal antibody (S2532, Sigma-Aldrich), a rabbit anti-TLR4 polyclonal antibody (sc-30002, Santa Cruz Biotecnology), or a rabbit anti-RAGE polyclonal antibody (ab3611, Abcam, Cambridge, UK) in 1% BSA in PBS with 0.1% Triton X-100. After washing with PBS, the sections were incubated with Alexa Fluor® 568-conjugated anti-chichen IgY (H+L) antibody (1:1000, A-11041, Thermo Fisher Scientific Corporation, Carlsbad, CA, USA), Alexa Fluor® 405-conjugated anti-mouse IgG (H+L) antibody (1:1000, A-31553, Thermo Fisher Scientific Corporation) and FITC-conjugated anti-rabbit IgG antibody (1:400, F7512, Sigma-Aldrich)

for 2 h at room temperature. The sections were washed, and then mounted by a cover slip with Fluoromount™ aqueous mounting medium (Sigma-Aldrich). Immunoreactivity was detected using a confocal laser fluorescence microscopy (FV10C-O, Olympus, Tokyo, Japan).

2.8. Statistics

Data are shown as the mean ± SEM. Statistical analysis was performed by Student's t-test for two-group data and by ANOVA followed by Tukey's-test for multiple comparisons. Significance was set at a $p < 0.05$ level.

3. Results

3.1 The anti-HMGB1-neutralizing antibody and rhsTM prevent the development of the chemotherapy-induced painful neuropathy in rats

Paclitaxel, when repeatedly administered at 2 mg/kg every 2 days, 4 times in total, caused neuropathic hyperalgesia in rats, the development of which was completely prevented by 7-time-repeated daily i.p. administration of the rat anti-HMGB1-neutralizing monoclonal antibody, but not the control IgG, at 1 mg/kg during paclitaxel treatment (Fig. 1a). Interestingly, 7-time-repeated local i.pl. administration of the anti-HMGB1 antibody, but not the control IgG, at 50 μg/paw, in the right hindpaw completely prevented the neuropathic hyperalgesia and allodynia in the ipsilateral hindpaw, although it exhibited only slight or no preventive effects in the contralateral hindpaw (Supporting information Fig. S1). Daily i.p. treatment with rhsTM at 10 mg/kg in the same time schedule as the antibody also prevented the development of the paclitaxel-induced neuropathic hyperalgesia in rats, an effect being dose-dependent in a range of 1-10 mg/kg (Fig. 1b).

Vincristine, when administered daily at 0.1 mg/kg for 5 days before and after a 2-day rest, 10 times in total, also caused neuropathic hyperalgesia (Fig. 2a and c) and allodynia (Fig. 2b and d) in rats, which were prevented by repeated daily i.p. administration of the rat

anti-HMGB1-neutralizing monoclonal antibody at 1 mg/kg (Fig. 2a and b) or rhsTM at 3-5 mg/kg, but not 1 mg/kg (Fig. 2c and d), during vincristine treatment.

3.2. Delayed single administration of the anti-HMGB1-neutralizing antibody or rhsTM temporally reverses the established chemotherapy-induced neuropathy in rats

We next evaluated if HMGB1 inactivation reverses the established neuropathy due to treatment with paclitaxel or vincristine in rats. The rat anti-HMGB1-neutralizing monoclonal antibody at 1 mg/kg, when administered i.p. once 14 days after the onset of paclitaxel treatment (Fig. 3a), reversed the neuropathic hyperalgesia (Fig. 3b). The deletion of HMGB1 by a single i.p. administration of rhsTM at 10 mg/kg 14 days after the onset of paclitaxel treatment (Fig. 3a) also reversed the neuropathic hyperalgesia (Fig. 3e). Interestingly, a single i.pl. administration of the rat anti-HMGB1 antibody at 50 μg/paw also reversed the paclitaxel-induced neuropathic hyperalgesia (Fig. 3c), an effect mimicked by the chicken anti-HMGB1-neutralizing polyclonal antibody, but not the control IgY, at the same dose (Fig. 3d). Similarly, a single i.p. administration of the rat anti-HMGB1 antibody (Fig. 4a and b) or rhsTM (Fig. 4c and d) 14 days after the onset of vincristine treatment dramatically reversed the neuropathic hyperalgesia and allodynia in rats.

3.3. Involvement of TLR4 and RAGE in the hyperalgesia induced by local (i.pl.) administration of HMGB1 or by chemotherapy in rats

HMGB1, when administered i.pl. at 10 μg/paw, caused slowly developing and long-lasting mechanical hyperalgesia (Fig. 5a and b), accompanied by the increased paw thickness (Fig. 5c), in agreement with our previous study (Tanaka et al. 2013). Given evidence for involvement of TLR4 and RAGE, known as targets of HMGB1, in pain processing (Agalave et al. 2014; Tanaka et al. 2014), we asked if blocking these receptors affects the HMGB1-induced hyperalgesia in rats. LPS-RS, a TLR4 antagonist, at 50 μg/paw significantly inhibited the decreased nociceptive threshold for 3-5 h after the i.pl. HMGB1 (Fig. 5b), but not the paw swelling at 5 h (Fig. 5c). Similarly, LMWH, known to inhibit RAGE (Liu et al. 2009; Myint et al. 2006; Takeuchi et al. 2013), at 10 μg/paw significantly reduced the i.pl. HMGB1-induced hyperalgesia, but not paw swelling (Fig. 5b and c).

In the rats with paclitaxel-induced neuropathy, i.pl. administration of LPS-RS or LMWH significantly suppressed the mechanical hyperalgesia (Fig. 5d). The vincristine-induced neuropathic hyperalgesia was transiently reversed by i.pl. LPS-RS (Fig. 5e), and in part, by i.pl. LMWH (Fig. 5f).

3.4. Effects of chemotherapy on serum HMGB1 levels and on HMGB1 and HDAC1/4 levels in

the sciatic nerves, DRG and hindpaw tissue of rats

Serum HMGB1 levels remained constant following the repeated treatment with paclitaxel or vincristine in rats (Fig. 6a). In contrast, HMGB1 protein levels in the L4-L6 DRG, but not the sciatic nerves or hindpaw tissue, dramatically decreased in the rats treated with paclitaxel or vincristine (Fig. 6b). Paclitaxel treatment significantly decreased the protein levels of HDAC4, but not HDAC1, in the DRG, and did not alter expression of HDAC1 or HDAC4 in the hindpaw tissue (Supporting information Fig. S2). Surprisingly, vincristine treatment caused great decreases in protein levels of HDAC1 and HDAC4 in both DRG and hindpaw tissues (Supporting information Fig. S2). Expression levels of RAGE or TLR4 in the sciatic nerves and DRG did not change after paclitaxel treatment (Fig. 6c).

3.5. Immunohistochemical analysis of HMGB1, RAGE and TLR4 in the sciatic nerves of rats with or without paclitaxel-induced neuropathy

In the sciatic nerves, HMGB1 (red), RAGE (green) and TLR4 (green) were detected in neurons or Schwann cells (blue), and HMGB1 was co-expressed with RAGE or TLR4 (yellow) (Fig. 7). The co-localization of HMGB1 with RAGE or TLR4 was found in some neurons and Schwann cells (white, as shown with arrows) (Fig. 7), which tended to increase

after paclitaxel treatment, particularly in Schwann cells (Fig. 7c, d).

4. Discussion

The present findings that inactivation of HMGB1 by the neutralizing antibody or rhsTM prevented and reversed the neuropathic hyperalgesia induced by paclitaxel or vincristine imply the roles of HMGB1 in both development and maintenance of chemotherapy-induced painful neuropathy. Given that the chemotherapy-induced neuropathic pain was also counteracted by LPS-RS or LMWH, our study suggests that TLR4 and RAGE in addition to HMGB1 might serve as therapeutic targets for clinical complication during and after cancer chemotherapy. We thus propose that HMGB1 inactivation, especially using rhsTM, during chemotherapy is clinically useful to prevent the development of neuropathic pain and improve the patients' quality of life.

In humans, rhsTM accelerates the formation of APC from protein C by thrombin, while it sequesters HMGB1 and then facilitates its degradation by thrombin (Ito and Maruyama 2011). These mechanisms appear to underlie the anti-coagulant and anti-inflammatory activity of rhsTM that is clinically used for treatment of DIC in Japan. In rodents, however, rhsTM is incapable of accelerating APC formation (Mohri et al. 1997), so that APC is not considered to contribute to the preventive or therapeutic effect of rhsTM on the chemotherapy-induced painful neuropathy in rodents. Considering that rhsTM cannot be administered orally but parenterally, the preventive application of rhsTM during chemotherapy might be much more

beneficial than its therapeutic use. The anti-HMGB1-neutralizing antibody, if humanized, might also be used for prevention of chemotherapy-induced peripheral neuropathy.

There is plenty of evidence for increases in total levels of HMGB1 protein in the spinal cord or DRG of rats with surgically induced neuropathic pain (He et al. 2013; Kuang et al. 2012; Nakamura et al. 2013; Shibasaki et al. 2010) or diabetic painful neuropathy (Ren et al. 2012). Nevertheless, it remains unclear whether the extracellular levels of HMGB1 released from cells including neurons actually increase in the spinal cord or DRG of those neuropathic pain models. In general, persistent release of HMGB1 would reduce the intracellular HMGB1 levels, and then cause no change or decrease in its total levels including both intracellular and extracellular HMGB1, unless HMGB1 is transcriptionally upregulated. Actually, we have reported that, in mice with cyclophosphamide-induced cystitis, the bladder pain is inhibited by inactivation of extracellular HMGB1, suggesting the involvement of increased HMGB1 release, and that they exhibited remarkable decreases in both total levels of HMGB1 protein in the bladder tissue and strength of HMGB1-like staining in the nucleus of urothelial cells, as assessed by Western blotting and immunohistochemistry, respectively (Tanaka et al. 2014). In the present study, since a single administration of the anti-HMGB1-neutralizing antibody reversed the chemotherapy-induced neuropathic pain, we consider that the continuous release of HMGB1 might maintain the neuropathic pain, leading to the decreased HMGB1 levels in DRG (see Fig. 6b). However, the most recent paper has demonstrated that mRNA for

HMGB1 is constitutively transported into axons and used for localized HMGB1 protein synthesis in cultured rat DRG neurons and that, in the rat exposed to sciatic nerve crush, HMGB1 protein levels increase in the sciatic nerve section proximal to the crush site, but decrease in DRG (Merianda et al. 2015). The authors thus propose that translation of axonal HMGB1 mRNA is enhanced after injury conditioning, followed by decreased HMGB1 levels in the cell body, and that the intraaxonally synthesized HMGB1 contributes to axonal growth. It is noteworthy that paclitaxel treatment even rather tended to elevate HMGB1 levels in the sciatic nerves, although it reduced HMGB1 levels in the DRG (see Fig. 6b). It remains to be confirmed whether the axonally generated HMGB1 is secreted and plays a role in an autocrine or paracrine fashion. This would be one of the most critical points to clarify the physiological role of HMGB1 in the sensory neurons and to understand our data in the present study. The peripheral axons (fiber) and endings of the primary afferents, and also their cell body in DRG could be their acting sites for HMGB1 in pain processing. Our study implies that peripheral HMGB1 released from the peripheral axon or ending of DRG neurons or from the neighboring non-neuronal cells might play important roles in the development of chemotherapy-induced painful neuropathy, since the anti-HMGB1-neutralizing antibody or rhsTM would not penetrate into the spinal dorsal horn because of their large molecular size. It is also supported by our results; i) repeated i.pl. injection of the anti-HMGB1-neutralizing antibody in the right hindpaw prevented the paclitaxel-induced hyperalgesia/allodynia in the

ipsilateral, but not contralateral, hindpaw (see Supporting information Fig. S1), ii) i.pl. injection of the antibody or blockers of TLR4 or RAGE reversed the chemotherapy-induced neuropathic pain (see Figs. 3c, 3d, 5d, 5e, 5f) and iii) i.pl. injection of HMGB1 produced mechanical hyperalgesia (see Fig. 5a).

Interestingly, paclitaxel treatment increases the number of activated macrophages in the DRG and peripheral nerves (Liu et al. 2010; Peters et al. 2007). Given that activated macrophages actively secrete HMGB1 (Malarkey and Churchill 2012; Yanai et al. 2012), the involvement of macrophage-derived HMGB1 in the paclitaxel-induced neuropathy should also be taken into consideration. The acetylated HMGB1 by HATs is translocated from the nuclear to the cytoplasm and released to the extracellular space, and the deacetylation of HMGB1 by HDACs such as HDAC1 and HDAC4 inhibits HMGB1 release (Evankovich et al. 2010). Therefore, it is likely that accelerated acetylation of HMGB1 might occur following the downregulation of HDAC1 and/or HDAC4 in the DRG (see Fig. 6c), leading to active secretion of HMGB1 from neurons or neighboring non-neuronal cells. The locally secreted HMGB1 might be too little to increase HMGB1 levels in the blood (see Fig. 6a), and/or the HMGB1 in the blood stream would be degraded by thrombin in association with thrombomodulin expressed in the endothelial cells. In future, we may have to examine HMGB1 levels in the cerebrospinal fluid after cancer chemotherapy, although peripheral HMGB1 rather appears to play a major role in the chemotherapy-induced neuropathy, as

mentioned above.

The amino acid sequence of HMGB1 is 99% identical among mammalian species, and there are three cysteine residues at potions 23, 45 and 106 within its molecule (Agalave and Svensson 2014). Nuclear HMGB1 exists in an all reduced form, known as all-thiol HMGB1, and, once released to the extracellular space, acts as a ligand for RAGE. The all-thiol HMGB1, when oxidized in the extracellular milieu, is converted into disulfide HMGB1 that has a disulfide bridge between two cysteine residues at positions 23 and 45, and is capable of activating TLR4, but not RAGE (Huang et al. 2010). There is evidence that both RAGE and TLR4 are expressed in immune cells located in the close vicinity of nociceptors and even in sensory neurons, and play a pronociceptive role (Agalave and Svensson 2014). In the present study, i.pl. administration of HMGB1, possibly in an all-thiol form, caused slowly developing and long-lasting hyperalgesia, in agreement with our previous study, which was significantly reduced by LMWH, known to inhibit RAGE, but also slightly attenuated by LPS-RS, a TLR4 antagonist (see Fig. 5), thereby suggesting that the injected HMGB1 might be partially converted into disulfide HMGB1 within the tissue. The distinct effectiveness of LMWH and LPS-RS in the rats treated with paclitaxel and vincristine (see Fig. 5d, e and f) indicates that those two anti-cancer drugs might brought about distinct redox states and disulfide HMGB1/all-thiol HMGB1 ratios in the peripheral tissue. The importance of the interaction of HMGB1 with TLR4 and RAGE is also supported by our immunohistochemical evidence for

co-localization of HMGB1 with TLR4 and RAGE in neurons and Schwann cells in the sciatic nerves (see Fig. 7). The well-described common downstream signal of TLR4 and RAGE targeted by HMGB1 is the NF-κB pathway (Venereau et al. 2013), which is involved in the pathogenesis of neuropathic pain (Niederberger and Geisslinger 2008). Apart from TLR4 and RAGE, it is still open to question if TLR2, CXCR4 and N-methyl-D-aspartate receptors, targeted by HMGB1 (Pedrazzi et al. 2012; Yanai et al. 2012), participate in chemotherapy-induced peripheral neuropathy.

It is easily understandable that i.pl. HMGB1-induced hyperalgesia takes hours to appear (see Fig. 5a), considering the downstream signals of the target molecules including TLR4 and RAGE which would include relatively long-lasting, transcriptional effects (Malarkey and Churchill 2012; Yanai et al. 2012). On the other hand, the rapid and transient anti-hyperalgesic effects of the anti-HMGB1 antibody, when administered after the establishment of neuropathy (see Figs. 3 and 4), do not seem to involve such long-lasting transcriptional effects. Most recently, it has been demonstrated that exogenously applied HMGB1 causes prompt increase in intracellular Ca^{2+} concentration and in the number of elicited action potentials within 3 min in isolated rat DRG nociceptor neurons (Allette et al. 2014; Feldman et al. 2012). Therefore, it is likely that continuously released HMGB1 might directly enhance the excitability of nociceptor neurons in the mice with chemotherapy-induced neuropathy. Nevertheless, it remains unclear why the anti-hyperalgesic

effect of the anti-HMGB1 antibody, given after the establishment of neuropathy, disappeared so quickly, although we speculate that unknown pronociceptive molecules might be activated and compensate the lack of HMGB1, reproducing hyperalgesia.

5. Conclusions

Our study strongly indicates the critical role of HMGB1 in both development and maintenance of chemotherapy-induced painful neuropathy, and suggests that inactivation of HMGB1 with the anti-HMGB1-neutralizing antibody or rhsTM is clinically useful to prevent and treat the neuropathic pain during and after chemotherapy. Our data also imply that RAGE and TLR4 serve as additional therapeutic targets for chemotherapy-induced painful neuropathy.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure legend

Fig. 1 Preventive effect of repeated i.p. administration of the rat anti-HMGB1-neutralizing antibody or rhsTM on the paclitaxel-induced neuropathic hyperalgesia in rats. Paclitaxel (PCT) at 2 mg/kg or vehicle (V) was repeatedly administered i.p. to the rat on day 0, 2, 4 and 6. The rat anti-HMGB1-neutralizing antibody (Rat anti-HMGB1) or the control IgG at 1 mg/kg or vehicle (a) and rhsTM at 1-10 mg/kg or vehicle (b) were administered i.p. once a day for 7 days during paclitaxel treatment, 7 times in total. The nociceptive threshold was determined by the paw pressure test. Data show the mean with S.E.M. for 4-5 (a) or 4-9 (b, left) rats and for 11-14 vehicle-treated and 4-6 rhsTM-treated rats (b, right). The baseline threshold for the paw pressure test was as follows: (a) 115.0 ± 1.173 g (n = 14); (b) 126.1 ± 1.622 g (n = 23). *P<0.05, **P<0.01 vs. V + V. ††P<0.01 vs. IgG + PCT or V + PCT.

Fig. 2 Preventive effect of repeated i.p. administration of the rat anti-HMGB1-neutralizing antibody or rhsTM on the vincristine-induced neuropathic hyperalgesia or allodynia in rats. Vincristine (VCR) at 0.1 mg/kg or vehicle (V) was repeatedly administered i.p. daily to the rat in two 5-day cycles with 2-day break between the cycles, 10 times in total. Rat anti-HMGB1-neutralizing antibody (Rat anti-HMGB1) or the control IgG at 1 mg/kg or vehicle (a and b) and rhsTM at 1, 3 or 5 mg/kg or vehicle (c and d) were administered i.p.

once daily during vincristine treatment, 10 times in total. The nociceptive threshold was determined by the paw pressure test (a and c) or von Frey test (b and d). Data show the mean with S.E.M. for 5-6 (a and b) or 6-8 (c and d) rats. The baseline threshold for the paw pressure test was as follows: (a) 168.2 ± 3.894 g (n = 17); (c) 183.9 ± 2.805 (n = 32). **P<0.01 vs. V + V. †P<0.05, ††P<0.01, †††P<0.001 vs. IgG + VCR or V + VCR.

Fig. 3 Effect of a single i.p. or i.pl. administration of the rat or chicken anti-HMGB1-neutralizing antibody and of rhsTM on the paclitaxel-induced neuropathic hyperalgesia in rats. (a) Drug administration schedules. Mice received a single administration of anti-HMGB1-neutralizing antibodies or rhsTM 14 days after the onset of paclitaxel (PCT) treatment. (b), (c), (d) and (e) Time-course of nociceptive threshold after single i.p. (b) and i.pl. (c) administration of the rat anti-HMGB1-neutralizing antibody (Rat anti-HMGB1) or the control IgG at 1 mg/kg (b) and 50 μg/paw (c), respectively, after i.pl. administration of the chicken anti-HMGB1-neutralizing antibody (Chicken anti-HMGB1) or the control IgY at 50 μg/paw (d), and after i.p. administration of rhsTM at 10 mg/kg or vehicle (e). The nociceptive threshold was determined by the paw pressure test. Data show the mean with S.E.M. for 6-7 (b), 5 (c), 4-6 (d), or 5-8 (e) rats. The baseline threshold for the paw pressure test was as follows: (b) 117.3 ± 1.890 g (n = 19); (c) 141.9 ± 2.360 g (n = 15); (d) 129.4 ± 5.818 g (n = 18); (e) 144.1 ± 3.776 g (n= 27). *P<0.05, **P<0.01 vs. V + V or V + IgY. †P<0.05,

††P<0.01 vs. PCT + V, PCT + IgG or PCT + IgY.

Fig. 4 Effect of a single i.p. administration of the rat anti-HMGB1-neutralizing antibody or rhsTM on the vincristine-induced neuropathic hyperalgesia or allodynia in rats. The rat anti-HMGB1-neutralizing antibody (Rat anti-HMGB1) or the control IgG at 1 mg/kg or vehicle (a and b) and rhsTM at 10 mg/kg or vehicle (c and d) were administered i.p. once 14 days or more after the onset of vincristine treatment. The nociceptive threshold was determined by the paw pressure test (a, c) or von Frey test (b, d). The baseline threshold for the paw pressure test was as follows: (a) 186.6 ± 4.026 g (n = 30); (c) 167.7 ± 1.377 g (n = 15). Data show the mean with S.E.M. for 8-11 (a, b) or 4-6 (c, d) rats. **P<0.01, ***P<0.001 vs. V + V. †P<0.05, ††P<0.01, †††P<0.001 vs. VCR + V or VCR + IgG.

Fig. 5 Effects of a single i.pl. administration of LPS-RS, a TLR4 antagonist, or LMWH, known to inhibit RAGE, on the hyperalgesia induced by i.pl. HMGB1 or the neuropathic hyperalgesia induced by treatment with paclitaxel or vincristine in rats. (a) The time course of mechanical nociceptive threshold after i.pl. HMGB1 at 10 μg/paw or vehicle (V) in rats. (b and c) Effects of i.pl. LPS-RS at 50 μg/paw or LMWH at 10 μg/paw on the mechanical hyperalgesia (b) or increased paw thickness at 5 h (c) caused by i.pl. HMGB1 at 10 μg/paw in rats. The rats received i.pl. LPS-RS or LMWH 30 min before i.pl. HMGB1. (d, e and f)

Effects of i.pl. LPS-RS or LMWH on the neuropathic hyperalgesia 14 days or more after the onset of treatment with paclitaxel (PCT) (d) or vincristine (VCR) (e, f) in rats. Data show the mean with S.E.M. for 4-5 (a), 10-12 (b, c), 4-6 (d, f) or 5-6 (e). The baseline threshold for the paw pressure test was as follows: (a) 194.3 ± 9.014 g (n = 9); (b) 161.5 ± 5.085 g (n = 44); (d) 116.5 ± 1.248 g (n = 18); (e) 181.6 ± 4.205 g (n = 17); (f) 168.4 ± 4.243 g (n = 34). *P<0.05, **P<0.01, ***P<0.001 vs. V or V + V. †P<0.05, ††P<0.01 vs. V + HMGB1, PCT + V or VCR + V.

Fig. 6 Protein levels of HMGB1, RAGE and TLR4 in the plasma, sciatic nerves, DRG or hindpaw of rats with neuropathy caused by treatment with paclitaxel or vincristine. Plasma HMGB1 levels (a) were determined by ELISA, and tissue HMGB1 (b) and RAGE or TLR4 levels (c) were quantified by Western blotting, 14 days or more after the onset of treatment with paclitaxel (PCT) or vincristine (VCR) or vehicle (V). Typical photographs of Western blotting are shown on the top (b, c). Data show the mean with S.E.M. for 4-6 rats (a, c) and for 7-12 (PCT) or 6 (VCR) rats (b). *P<0.05 vs. V.

Fig. 7 Immunohistochemical localization of HMGB1, RAGE and TLR4 in the sciatic nerves of rats with paclitaxel-induced neuropathy. NF200 (blue), a neuron marker (a, b) or S100β (blue), a Schwann cell marker (c, d), and HMGB1 (red) were co-stained with RAGE

(green) (a, c) or TLR4 (green) (b, d) in the sciatic nerves of rats treated with vehicle (V) or paclitaxel (PCT). Merges of HMGB1/RAGE (yellow) (a, c) or HMGB1/TLR4 (yellow) (b, d) and of HMGB1/RAGE/NF200 (white) (a), HMGB1/TLR4/NF200 (white) (b), HMGB1/RAGE/S100β (white) (c) or HMGB1/TLR4/S100β (white) (d). Bars show 10 μm. Arrows indicate co-localization of HMGB1 with RAGE or TLR4 in neurons or Schwann cells.

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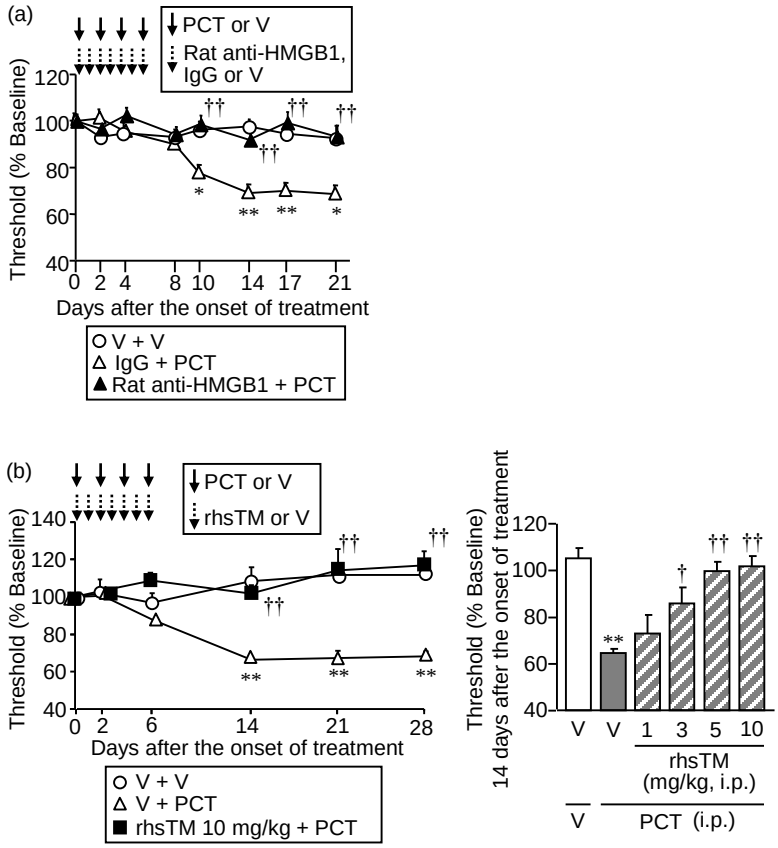

(a)
PCT or V
Rat anti-HMGB1, IgG or V
Threshold (% Baseline)
120
100
80
60
40
0 2 4 8 10 14 17 21
Days after the onset of treatment
V + V
IgG + PCT
Rat anti-HMGB1 + PCT
(b)
PCT or V
rhsTM or V
Threshold (% Baseline)
140
120
100
80
60
40
0 2 6 14 21 28
Days after the onset of treatment
V + V
V + PCT
rhsTM 10 mg/kg + PCT
Threshold (% Baseline) 14 days after the onset of treatment
120
100
80
60
40
V V 1 3 5 10
rhsTM (mg/kg, i.p.)
V
PCT (i.p.)

Fig. 1

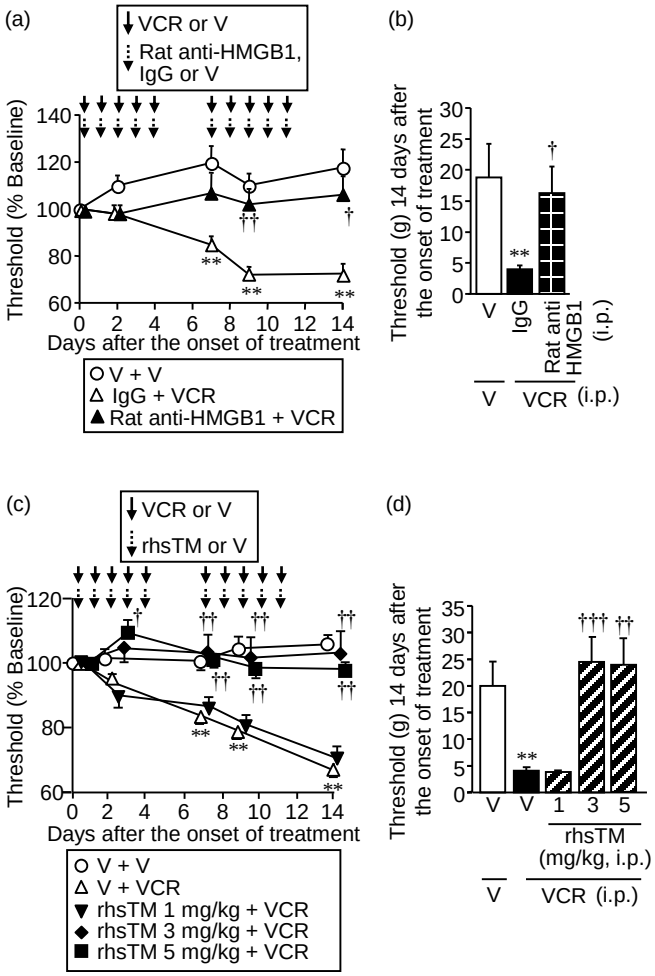

(a)
VCR or V
Rat anti-HMGB1, IgG or V
Threshold (% Baseline)
Days after the onset of treatment
V + V
IgG + VCR
Rat anti-HMGB1 + VCR
(b)
Threshold (g) 14 days after the onset of treatment
V
IgG
Rat anti HMGB1 (i.p.)
V
VCR (i.p.)
(c)
VCR or V
rhsTM or V
Threshold (% Baseline)
Days after the onset of treatment
V + V
V + VCR
rhsTM 1 mg/kg + VCR
rhsTM 3 mg/kg + VCR
rhsTM 5 mg/kg + VCR
(d)
Threshold (g) 14 days after the onset of treatment
rhsTM (mg/kg, i.p.)
V
VCR (i.p.)

Fig. 2

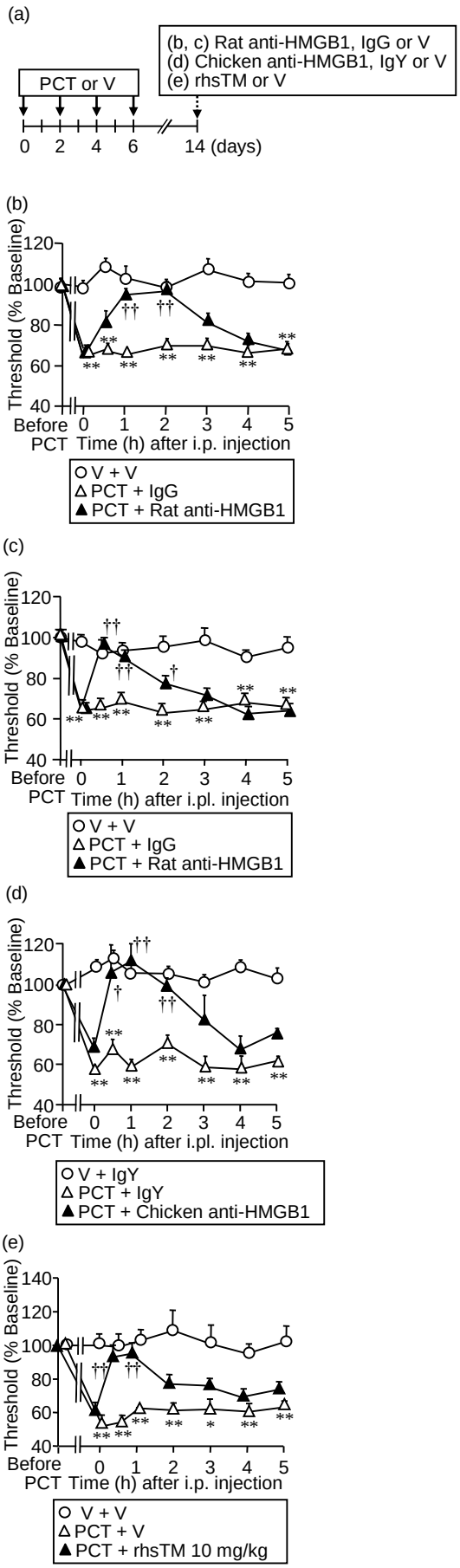

(a)
(b, c) Rat anti-HMGB1, IgG or V
(d) Chicken anti-HMGB1, IgY or V
(e) rhsTM or V
PCT or V
0 2 4 6 14 (days)
(b)
Threshold (% Baseline)
Before PCT
Time (h) after i.p. injection
V + V
PCT + IgG
PCT + Rat anti-HMGB1
(c)
Threshold (% Baseline)
Before PCT
Time (h) after i.pl. injection
V + V
PCT + IgG
PCT + Rat anti-HMGB1
(d)
Threshold (% Baseline)
Before PCT
Time (h) after i.pl. injection
V + IgY
PCT + IgY
PCT + Chicken anti-HMGB1
(e)
Threshold (% Baseline)
Before PCT
Time (h) after i.p. injection
V + V
PCT + V
PCT + rhsTM 10 mg/kg

Fig. 3

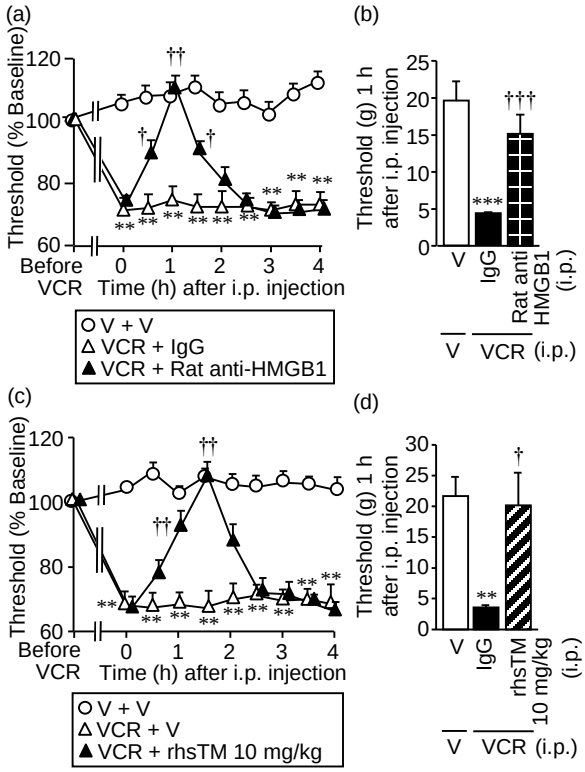

(a)
Threshold (% Baseline)
120
100
80
60
Before VCR
0
1
2
3
4
Time (h) after i.p. injection
V + V
VCR + IgG
VCR + Rat anti-HMGB1
(b)
Threshold (g) 1 h after i.p. injection
25
20
15
10
5
0
V
IgG
Rat anti HMGB1 (i.p.)
V
VCR (i.p.)
(c)
Threshold (% Baseline)
120
100
80
60
Before VCR
0
1
2
3
4
Time (h) after i.p. injection
V + V
VCR + V
VCR + rhsTM 10 mg/kg
(d)
Threshold (g) 1 h after i.p. injection
30
25
20
15
10
5
0
V
IgG
rhsTM 10 mg/kg (i.p.)
V
VCR (i.p.)

Fig. 4

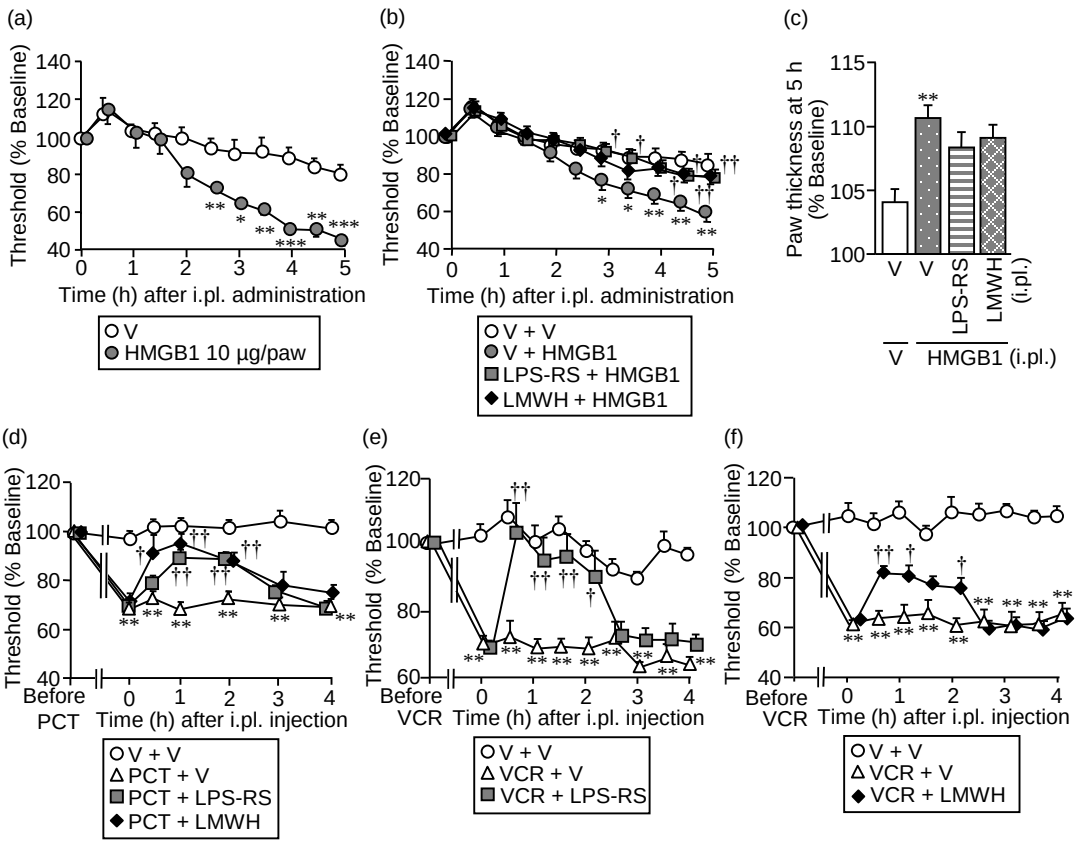

(a)
Threshold (% Baseline)
Time (h) after i.pl. administration
V
HMGB1 10 µg/paw
(b)
Threshold (% Baseline)
Time (h) after i.pl. administration
V + V
V + HMGB1
LPS-RS + HMGB1
LMWH + HMGB1
(c)
Paw thickness at 5 h (% Baseline)
V
V
LPS-RS
LMWH
(i.pl.)
V
HMGB1 (i.pl.)
(d)
Threshold (% Baseline)
Before PCT
Time (h) after i.pl. injection
V + V
PCT + V
PCT + LPS-RS
PCT + LMWH
(e)
Threshold (% Baseline)
Before VCR
Time (h) after i.pl. injection
V + V
VCR + V
VCR + LPS-RS
(f)
Threshold (% Baseline)
Before VCR
Time (h) after i.pl. injection
V + V
VCR + V
VCR + LMWH

Fig. 5

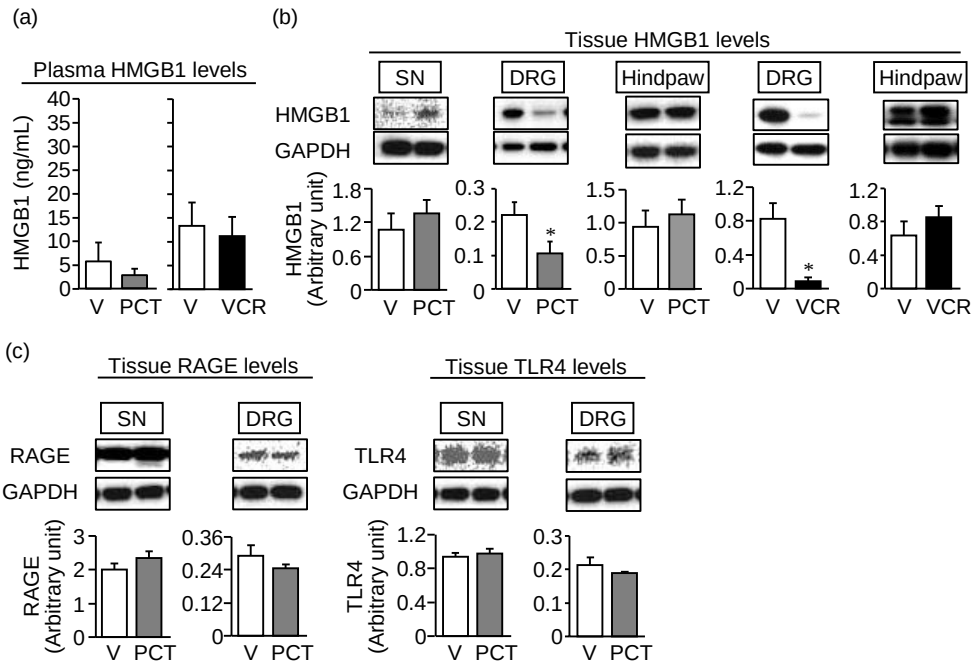

(a)
Plasma HMGB1 levels
HMGB1 (ng/mL)
40
35
30
25
20
15
10
5
0
V PCT V VCR
(b)
Tissue HMGB1 levels
SN
DRG
Hindpaw
DRG
Hindpaw
HMGB1
GAPDH
HMGB1 (Arbitrary unit)
1.8
1.2
0.6
0
0.3
0.2
0.1
1.5
1.0
0.5
1.2
0.8
0.4
V PCT
V PCT
V PCT
V VCR
V VCR
*
*
(c)
Tissue RAGE levels
Tissue TLR4 levels
SN
DRG
SN
DRG
RAGE
GAPDH
TLR4
GAPDH
RAGE (Arbitrary unit)
3
2
1
0
0.36
0.24
0.12
TLR4 (Arbitrary unit)
1.2
0.8
0.4
0.3
0.2
0.1
V PCT
V PCT
V PCT
V PCT

Fig. 6

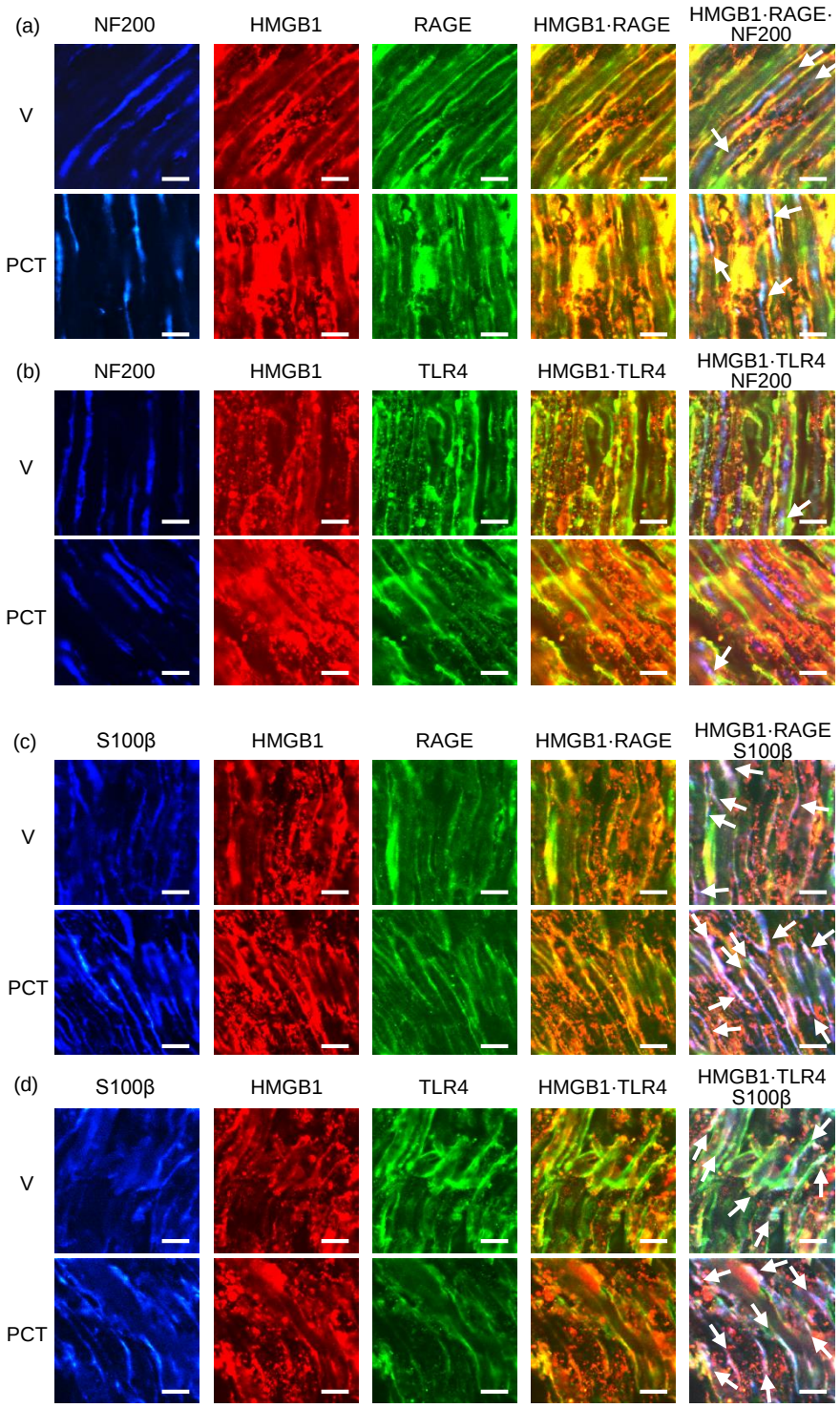
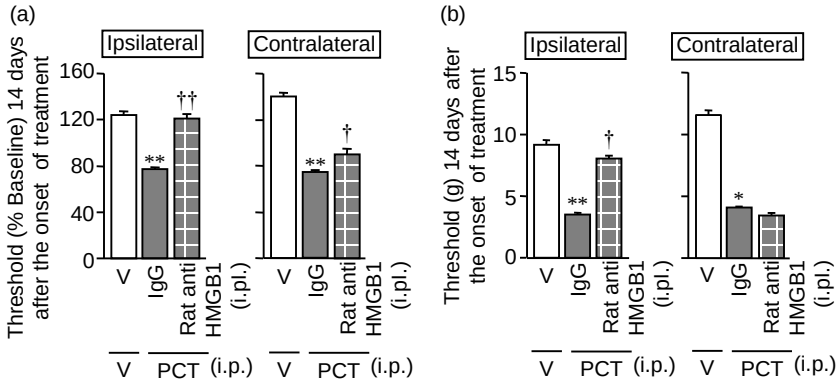
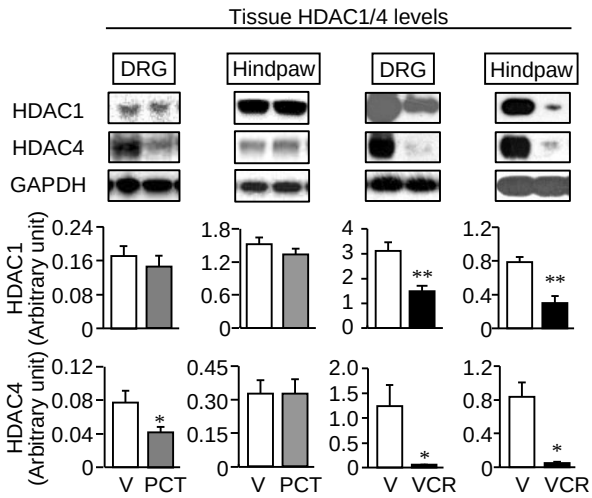

(a)
NF200
HMGB1
RAGE
HMGB1·RAGE
HMGB1·RAGE·
NF200
V
PCT
(b)
NF200
HMGB1
TLR4
HMGB1·TLR4
HMGB1·TLR4
NF200
V
PCT
(c)
S100β
HMGB1
RAGE
HMGB1·RAGE
HMGB1·RAGE
S100β
V
PCT
(d)
S100β
HMGB1
TLR4
HMGB1·TLR4
HMGB1·TLR4
S100β
V
PCT

Fig. 7



Supplementary Figure 1. Effect of repeated i.pl. administration of the rat anti-HMGB1 neutralizing antibody on the paclitaxel-induced neuropathic hyperalgesia or allodynia in rats. Paclitaxel (PCT) at 2 mg/kg or vehicle (V) was repeatedly administered i.p. on day 0, 2, 4 and 6. The rat anti-HMGB1 neutralizing antibody at 50 μg/paw or the control IgG was repeatedly administered i.pl. in the right hindpaw once a day for 7 days during paclitaxel treatment, 7 times in total. The nociceptive threshold in the ipsilateral and contralateral hindpaws was evaluated by the paw pressure test (a) or von Frey test (b). Data show the mean with S.E.M. for 5 rats. *P<0.05, **P<0.01 vs. V+V. †P<0.05, ††P<0.01 vs. PCT+IgG.



Supplementary Figure 2. The protein levels of HDAC1 or HDAC4 proteins in the DRG or hindpaw of rats with neuropathy caused by treatment with paclitaxel or vincristine. Tissue HDAC1 or 4 levels were determined by Western blotting 14 days or more after the onset of treatment with paclitaxel (PCT) or vincristine (VCR) or vehicle (V). Typical photographs of Western blotting are shown on the top. Data show the mean with S.E.M. for 7-12 (PCT) or 3-6 (VCR). *P<0.05, **P<0.01 vs. V.