Research article

Activation of spinal macrophage-inducible C-type lectin induces mechanical allodynia and microglial activation in rats

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ABSTRACT

Macrophage-inducible C-type lectin (Mincle), a pattern recognition receptor, is a critical component of the innate immune system that is involved in the pathogenesis of chronic pain. Previous studies have reported the expression of Mincle in neuronal and glial cells of the brain, but its expression and role in pain processing at the spinal level remain to be determined. The current study was performed to identify Mincle in the spinal cord and to investigate the effect of Mincle activation on spinal sensitization.

Most Mincle immunoreactivity was localized within the grey matter and the dorsal and ventral horns of the lumbar spinal cord in naïve rats. A single intrathecal (i.t.) injection of trehalose-6,6-dibehenate (TDB), a Mincle ligand, induced mechanical allodynia. Immunoreactivity to Mincle and Iba-1 in the spinal cord significantly increased after i.t. injection of TDB. Mechanical allodynia was attenuated by daily i.t. injection of minocycline. However, double immunofluorescence revealed that Mincle co-localizes with NeuN (neurons), but not with Iba-1 (microglia) or GFAP (astrocytes).

In conclusion, we found that Mincle was present in spinal cord neurons, but not microglia or astrocytes, and may play a role in microglia-induced spinal sensitization.

1. Introduction

A great deal of evidence suggests that immune reactions play an important role in the development of hypersensitivity, which is an essential feature of neuropathic pain [3,9,10,21]. Such reactions generally involve proinflammatory cytokines released by neuroglia as well as classical immunocompetent cells including macrophages or T-lymphocytes. The involvement of immune system cells in pathological pain is not confined to peripheral nerves but is also evident at the spinal level [4,17,23].

Pattern recognition receptors (PRRs) are critical components of the innate immune system, recognizing molecules characteristic of microbial pathogens and dead or damaged native cells, presenting them as antigens [18]. PRRs are expressed in peripheral immune cells as well as microglia, astrocytes, and neurons of the peripheral and central nervous systems [11]. Toll-like receptors (TLRs), a PRR family, have been investigated extensively in terms of involvement in the development of persistent pain [11,14], but little is known about the role played by C-type lectin receptors (CLRs; another major PRR family of the immune system) in sensitization at the spinal level.

Macrophage-inducible C-type lectin (Mincle) is a CLR expressed principally in macrophages [15]. The Mincle ligand interacts with the Fc receptor common γ-chain (the Fc gamma receptor) to induce intracellular signaling by spleen tyrosine kinases, which in turn trigger the release of proinflammatory cytokines and chemokines [13,22]. Mincle is expressed in the microglia and neurons of rodent and human brains [5,8,16]. Mincle is upregulated after traumatic brain injury, ischemic stroke, and subarachnoid hemorrhage. Although its presence did not affect outcomes in an animal model of spinal cord injury [2], congenic rats expressing CLRs including Mincle exhibited a stronger inflammatory response and longer pain duration after peripheral nerve injury [6]. Here, we identified Mincle in the rat spinal cord and explored its role in spinal sensitization.

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2. Materials and Methods

2.1. Animals

All experiments were performed in accordance with the International Association for the Study of Pain guidelines for the Use of Animals in Research. The protocol was approved by our Institutional Animal Care and Use Committee (approval no. CNU IACUC-H-2017-12). Male Sprague-Dawley rats weighing 225–250 g were housed at a constant temperature of 22–23 °C under an alternating 12-h/12-h light/dark cycle with free access to food and water.

2.2. Intrathecal administration of Mincle ligand or minocycline

A polyethylene-5 catheter was implanted into the intrathecal (i.t.) space to allow experimental drug administration, as described previously [12]. Under general anesthesia induced by sevoflurane, the catheter was introduced through the atlanto-occipital membrane and advanced caudally by 8.5 cm to the level of lumbar enlargement. Any rats exhibiting neurological deficits after catheter implantation were immediately sacrificed by an overdose of inhalational anesthetic. All animals were housed in individual cages after surgery. After a recovery period of 5 days, the animals were used in experiments.

Trehalose-6,6-dibehenate (TDB, InvivoGen), a synthetic Mincle ligand, was dissolved in 10% (v/v) dimethyl sulfoxide and then delivered to the i.t. space (10 μL), followed by 10 μL normal saline (to flush the catheter) using a hand-driven, geared, syringe pump. i.t. administration of TDB or vehicle was performed only once. In some animals, i.t. administration of 60 μg minocycline (60 μg) was performed only once. In some animals, i.t. administration of TDB or vehicle was performed only once. In some animals, i.t. administration of 60 μg minocycline (60 μg) was performed only once. In some animals, i.t. administration of TDB or vehicle was performed only once.

2.3. Behavioral tests

The paw withdrawal threshold (PWT) was measured by application of calibrated von Frey filaments (Stoelting) to the hind paw. The PWT with 50% probability was calculated using the up-and-down method. If the animal did not exhibit withdrawal or a licking response, it was assigned a 15-g PWT (the cut-off value). Rats were randomly assigned to the vehicle or TDB group, and received an i.t. injection of vehicle or TDB at the maximal soluble dose (10 μg). All investigators were blinded to the treatments.

On the day of i.t. injection (day 0), PWT was measured 30 min prior to i.t. TDB administration (baseline PWT) and then every hour thereafter for 4 h to evaluate acute effects. Commencing the day after i.t. injection, PWT was measured twice at intervals of 10 min, and the average was calculated. After injection of minocycline or vehicle, measurements were obtained 1 h later. The PWT data are also presented as the area under the curve (AUC) for hyperalgesia, with days after i.t. injection plotted against the percentage of hyperalgesic effects calculated using the formula: [(baseline PWT - post-treatment PWT)/baseline PWT] × 100). The AUC is the sum of the area below the baseline, indicating the extent of mechanical allodynia. A greater AUC reflected a more intense allodynic response.

2.4. Immunofluorescence

Rats were deeply anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine (4:1 v/v, 0.7 mL/kg) and transcardially perfused with 200 mL 0.01 M phosphate-buffered saline (pH 7.4) followed by 300 mL 4% (v/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, the lumbar enlargements were harvested and post-fixed in the same perfusate at 4 °C for 6 h. The tissues were immersed in 20% (w/v) sucrose overnight (in which the tissues sank to the bottom, affording cytoprotection). After snap-freezing, transverse sections (15 μm thick) were obtained using a cryostat. All sections were blocked in 3% (v/v) normal chicken serum in 0.3% (v/v) Triton X-100 for 30 min at room temperature and subsequently incubated with a rabbit anti-Mincle receptor polyclonal antibody conjugated to Alexa Fluor 488 (1:200, Antibodies-online), mouse anti-Iba-1 monoclonal antibody (1:200, Abcam), mouse anti-NeuN monoclonal antibody (1:1,000, Millipore), or rabbit anti-GFAP polyclonal antibody (1:1,000, Dako) at 4 °C overnight. Antibody binding to tissue sections was visualized after incubation with chicken anti-mouse IgG or chicken anti-rabbit IgG conjugated to Alexa Fluor 488 (1:1,000, Invitrogen) for 2 h at room temperature. The sections were rinsed in 0.01 M phosphate-buffered saline, and cover slips were applied.

Bilateral images were captured using a fluorescence microscope (Olympus) at 10–60× and imported into the Adobe Photoshop software. To compare the fluorescence intensities, images were then converted to grayscale and analyzed using the Image J software. After drawing an outline of the dorsal horn gray matter, fluorescence intensity in the outline was obtained for each image and distinguished from the background intensity.

For double-label immunofluorescence evaluation, spinal cord sections were incubated in 3% (v/v) normal chicken serum for 30 min at room temperature and subsequently with rabbit anti-Mincle polyclonal antibody conjugated to Alexa Fluor 488 (1:200, Antibodies-online) at 4 °C overnight. Then, the tissue sections were incubated with the following antibodies: mouse anti-Iba-1 monoclonal antibody (1:200, Abcam), mouse anti-NeuN monoclonal antibody (1:1,000, Millipore) or goat anti-GFAP polyclonal antibody (1:200, Abcam) for 3 h at room temperature. Antibody-binding to tissue sections was visualized after incubating with chicken anti-mouse IgG conjugated to Alexa Fluor 594 (1:1,000, Invitrogen) or chicken anti-goat IgG conjugated to Alexa Fluor 594 (1:1,000, Invitrogen) for 1 h at 37 °C.

After confirming the co-localization of Mincle with NeuN, a square box with 25-μm-long sides was randomly placed on the dorsal and ventral horns in the NeuN and Mincle double immunostaining images. The number of NeuN-positive neurons co-localized with Mincle in the box was expressed as a proportion of the total number of NeuN-positive neurons in the box. This procedure was performed twice for each dorsal and ventral horn in four sections per rat, in a total of six animals.

2.5. Statistical analysis

PWT data are expressed as the mean ± standard error of the mean and the other data as the mean ± standard deviation. The overall differences in the changes of PWTs among groups were compared by repeated-measures analysis of variance (ANOVA). Differences in PWT on each day among groups were compared using a dependent samples t-test with Bonferroni correction or one-way ANOVA. Other data including the AUCs for hyperalgesia were analyzed by independent samples t-tests or one-way ANOVA followed by Tukey’s post-hoc comparison test using SPSS software. A P-value < 0.05 was considered to reflect statistical significance.

3. Results

A single i.t. injection of TDB induced mechanical allodynia as revealed by the von Frey test; the PWT began to decrease in test (compared with control) animals on day 1 (Fig. 1). Mechanical allodynia then became more intense; the PWT was lowest on day 3 and then gradually recovered to approximately baseline value by day 10. Furthermore, the AUC, indicating the extent of mechanical allodynia, was significantly greater in the TDB group (by more than four-fold) than in the vehicle group. However, no change in the PWT was observed during the 4 h immediately after TDB injection (data not shown), indicating that Mincle activation did not exert an acute effect.
Most Mincle immunoreactivity was located in the grey matter and the dorsal and ventral horns of the lumbar spinal cord in naïve rats. In the dorsal horn, Mincle was diffusely distributed throughout laminae I–V but was most densely expressed in laminae I and II (Figs. 2–4). On day 3, NeuN (Fig. 3C) and GFAP (Fig. 3D) immunoreactivity did not differ significantly between the TDB and control groups. However, the number of Iba-1-positive cells and Iba-1 fluorescence intensity were significantly elevated compared with the vehicle group (Fig. 3B). Furthermore, the intensity of Mincle staining increased after i.t. TDB (Fig. 3A).

Although the immunofluorescence data suggest that microglia were activated after i.t. TDB, the double immunofluorescence study revealed that Mincle does not co-localize with Iba-1 or GFAP (Figs. 4B, C). Rather, most Mincle staining was found in NeuN-positive cells, indicating that Mincle was confined to spinal cord neurons in naïve rats (Fig. 4A). In the dorsal horn, 96.5 ± 4.8% of NeuN-positive neurons were co-localized with Mincle, and all neurons expressed Mincle in the ventral horn. In rats given TDB i.t., the co-localization pattern of Mincle did not different from that of naïve rats (data not shown).

Mechanical allodynia was attenuated by daily i.t. administration of minocycline from days 0 to 10. The AUC for hyperalgesia was increased significantly in the minocycline group compared with the i.t. vehicle and TDB groups (Fig. 5).

4. Discussion

Mincle is a PRR that plays an important role in innate immunity by recognizing pathogens or dead self-cells. The PRRs are divided into four families, including the CLR and TLR families [18]. PRRs are expressed not only in macrophages and dendritic cells, but also by other immune and non-immune cells [11]. As a member of the CLR family, Mincle is a transmembrane protein that binds to carbohydrates in a Ca²⁺-dependent manner and is expressed principally in monocytes, macrophages, and dendritic cells involved in the innate inflammatory immune system [15].

We found that Mincle was expressed in the dorsal and ventral horns of the rat lumbar spinal cord, exclusively in neurons (thus not in microglia or astrocytes). Neuronal Mincle expression has been observed in rodent models of traumatic brain injury, subarachnoid hemorrhage, and ischemic stroke [5,8,16]. In those studies, Mincle was not confined to neurons but was expressed principally in microglia and endothelial cells [8,16]. We found that Mincle did not co-localize with Iba-1 (a microglial marker), which was surprising given that microglia are the resident macrophages of the CNS and Mincle is a myeloid receptor [7,22]. Although we cannot explain this finding, Mincle expression in brain microglia may differ depending on the injury model used and the animal species. Mincle was expressed in both Iba-1-positive microglia and neurons in a rat model of subarachnoid hemorrhage [8] and showed co-localization with Iba-1 in a mouse ischemic stroke model [16]. In the cited study, however, Mincle also co-localized with CD11b,
a marker of both microglia and macrophages. Furthermore, Mincle was expressed in both Iba-1- and CD11b-positive cells in human brain tissue of a patient with an infarction. Consistent with previous studies, we found that Mincle was not expressed in astrocytes. However, the extent of Mincle expression in the CNS remains unclear. A recent study showed that Mincle was expressed in perivascular macrophages but was not co-localized with neurons, glial cells, or pericytes in a model of CNS ischemia/reperfusion injury induced by transient middle cerebral artery occlusion in mice [2]. The cited authors did not report Mincle expression in the spinal cord.

Increased expression of both Mincle and the endogenous ligand SAP130 was evident in the animal models, including traumatic brain injury, subarachnoid hemorrhage, and ischemic stroke, of the aforementioned studies [5,8,16]. Mincle expression was also increased in brain tissue and/or cerebrospinal fluid of patients with traumatic brain injuries or infarction [5,16]. The neurological score, infarct volume, and brain water content all improved after inhibition of Mincle by knockdown or neutralizing antibody application, or inhibition of Mincle-downstream targets. In contrast, direct Mincle activation by exogenous recombinant SAP130 upregulated Mincle downstream signaling by the Syk/caspase-associated recruitment domain and the production of proinflammatory cytokines (interleukin-1β and tumor necrosis factor) [5,8]. In the current study, Mincle expression increased after i.t. TDB injection, which is consistent with observations of traumatic brain injury, subarachnoid hemorrhage, and ischemic stroke.

PRRs, critical components of innate immunity, play important roles in the pathogenesis of pain [11]. TLRs (representative PRRs) are present in neurons and glial cells of the nervous system and in peripheral immune cells of rodents and humans. Many studies have suggested that the TLRs of microglia, astrocytes, and neurons are involved in central sensitization, although the involvement of neuronal PRRs in such sensitization has been shown in only a few in vitro studies. However, preclinical studies have shown that PRRs may be useful therapeutic targets in patients with chronic pain.

In contrast, little is known about Mincle expression at the spinal level or its role in pain processing. Mincle knockout mice did not exhibit improved locomotor recovery or reduced lesion size in a model of contusive spinal cord injury, but neither Mincle expression per se nor the cell type(s) expressing Mincle was investigated [2]. In terms of spinal sensitization, one study explored the effect of CLR gene

Fig. 3. Changes in the expression levels of Mincle, Iba-1, NeuN, and GFAP in the dorsal horn of the lumbar spinal cord on day 3 after intrathecal injection of vehicle or TDB. The immunoreactivities of Iba-1 (A) and Mincle (B) increased significantly in the dorsal horn of TDB-treated rats compared with vehicle-treated rats, but no changes were observed in NeuN or GFAP levels (C, D). Sections were obtained from six animals per group. Scale bar: 25 μm. *P < 0.05, **P < 0.01 vs. Vehicle (independent samples t-test).
expression on pain susceptibility; congenic rats expressing four CLR genes including Mincle exhibited hyperalgesia (after peripheral nerve injury) of longer duration than did controls [6]. In the former animals, the levels of interleukins, substance P, and cathepsin S were elevated, accompanied by increased macrophage infiltration into the spinal cord.

In the present study, Mincle activation by TDB induced both pain behavior and spinal cord microglial activation. Although Mincle did not co-localize with microglia, microglia were activated after i.t. TDB, and pretreatment with i.t. minocycline attenuated the intensity of allodynia. Thus, microglia may be indirectly activated by Mincle signaling in neurons which can induce the activation of nuclear factor kappa B and increase the release of proinflammatory cytokines including tumor necrosis factor.

As a limitation of the current study, the role of Mincle was not evaluated in a specific animal model of pain. Several reports using different models of chronic pain found that endogenous PRR ligands...
such as danger-associated molecular pattern molecules (including HMGB1 and S100beta released from damaged cells) were up-regulated and released into the spinal cord without directly damaging the cord [1,19,20]. The molecular and cellular changes in the spinal cord following i.t. injection of TDB may be similar to those observed in such pain models. However, further in vivo studies are required to elucidate the signaling pathway and mechanism by which Mincle activation induces mechanical allodynia and microglial activation.

In conclusion, we found that Mincle was present in spinal cord neurons, but not microglia or astrocytes, and possibly played a role in microglia-induced spinal sensitization.

Conflict of interests
None to declare

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