Anti-high-mobility group box 1 Neutralizing Antibody Ameliorates Pain Hypersensitivity Induced by Intraplantar Administration of Complete Freund’s Adjuvant in Rats

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Abstract

Mechanisms underlying inflammation-induced pain remain elusive, but research has shown that inflammatory cytokines and immune responses in the spinal cord are especially involved. First reported as a nonhistone chromosomal protein, high-mobility group box 1 (HMGB1) is now implicated as a novel proinflammatory cytokine and crucial mediator of inflammation. We hypothesized that HMGB1 could trigger the release of cytokines in the spinal cord and contribute to inflammatory pain (IP). To test this hypothesis, we first built an IP model induced by intraplantar administration of complete Freund’s adjuvant (CFA) in rats. Moreover, an anti-HMGB1 antibody was injected intrathecally 1, 4, and 14 days after the adjuvant was administered. Pain behavioral responses were measured using a series of tests, and the expressions of spinal HMGB1, interleukin-1 beta (IL-β), and tumor necrosis factor alpha (TNF-α) were assessed. We found that intrathecal injection of the anti-HMGB1 antibody could effectively alleviate the behavioral hypersensitivity and reduce the expressions of spinal HMGB1, IL-β and TNF-α in IP rats. These results suggest that HMGB1 plays an important role in the development of IP induced by intraplantar administration of complete Freund’s adjuvant. HMGB1 blocking therapy holds potential in the treatment of IP.

Introduction

Inflammatory pain (IP) resulting partly from peripheral tissue injury and inflammation is characterized by unpleasant and persistent increases in pain hypersensitivity, including hyperalgesia and allodynia[1]. Prolonged suffering from pain induced by inflammatory processes can become a serious burden for the affected individuals, for instance, by impairing their ability to work and causing sleep disturbance, anxiety, and depression[2].

Inflammatory cytokines and immune responses in the spinal cord are especially involved in the pathogenesis process of IP[3]. Pro-inflammatory cytokines were reported that could promote central nervous system (CNS) immune cascade responses[4]. Copious pain-related mediators that can sensitize and lower the threshold of neuronal firing are produced: the pathology correlates with central sensitization and chronic pain states, eventually leading to hypersensitivity. The current pharmacotherapy for pain is still mainly based on steroidal or nonsteroidal anti-inflammatory drugs, and opioids are the most widely used treatment drugs for chronic pain states[5]. In fact, long-term use of drugs with adverse side effects poses a major dilemma in their clinical management, such as gastric ulcer as well as kidney and liver toxicity[6]. In recent years, much research has demonstrated that opioids promote the release of proinflammatory cytokines, and even induced hyperalgesia and allodynia following chronic opioid administration[7]. These data clearly indicate the need to develop less harmful analgesic/anti-inflammatory strategies for pain relief.

High-mobility group box 1 (HMGB1) is a nonhistone nuclear protein and belongs to HMG family and expressed in almost all cells and has been reported that played a pivotal role in several pathologies, such as sepsis, collagen disease, cancers, arthritis and systemic inflammation[8]. HMGB1 can promote...
progression of inflammatory immune responses to infection or injury[9]. It can also amplify the inflammation by enhances the secretion of proinflammatory cytokines and triggers a wide range of downstream effects through activating monocytes, macrophages and dendritic cells[10]. In addition, HMGB1 inhibit the phagocytosis of apoptotic neutrophils by macrophages via interactions with phosphatidylserine on the cell surface, which may retard the process of inflammation and enhance inflammatory responses[11]. Studies have shown that intracerebroventricular or intrathecal application of HMGB1 can induce an increase in the expressions of TNF and IL-6 expression in the brain and produce mechanical allodynia[12]. Moreover, administration of an anti-HMGB1 neutralizing antibody can create an effective and reliable anti-allodynia effect in models of neuropathic pain and bone cancer pain[13]. Interestingly, the physiological functions of HMGB1 in the spinal cord remain inadequately understood in the pathophysiological processes associated with IP.

In this study, we used a complete Freund's adjuvant (CFA)-induced IP model of rats to detect the time-course expression of spinal HMGB1. The animals were intrathecally injected with a neutralizing antibody against HMGB1, and their behavioral responses were measured. Furthermore, the expressions of spinal IL-1β and TNF-α were detected at the mRNA level. The results suggest that spinal HMGB1 is correlated with inflammatory hyperalgesia and allodynia. The new data enhances our understanding of the pathogenesis of IP and could be provide a new therapeutic target for the treatment of IP.

**Methods**

**Animals**

Unmated male Sprague-Dawley rats (weight, 180–220 g) provided by the Experimental Animal Center of Soochow University were used in this study. The animals were housed under a 12 h light/12 h dark cycle regime (lights on at 8:00 AM, lights off at 8:00 PM), at a constant room temperature of 22–24°C and with relative humidity of 40%-60%; food and water were provided libitum. Animal treatments were performed based on the guidelines established by the International Association for the Study of Pain[14].

**IP model**

IP was induced by intraplantar administration of CFA (100 µl into the surface of the left hind paw) suspended in an oil / saline (1:1) emulsion. The rats assigned to the sham group received 100 µl of normal saline.

Five days before the intraplantar injection of CFA or normal saline, in the rats under anesthesia with pentobarbital sodium (40 mg/kg, i.p.), a polyurethane intrathecal catheter (PE-10 tube) was inserted into their lumbar subarachnoid space through the L4-L5 intervertebral disc space. The catheter was tunneled subcutaneously and externalized through the skin in the neck region. The recovery course of the cannulated rats lasted 3–4 days. The volume of dead space of the intrathecal catheter was 10 µl. Rats that showed any impaired movement, neurological deficits or infection due to the surgical procedure were
excluded from analysis. The location of distal range was confirmed by the injection of pontamine sky blue via the catheter at the end of all experiments.

**Intrathecal injection**

A polyclonal neutralizing antibody against HMGB1 (20 µg/10µl, SHINO, Japan) or vehicle control IgG (20 µg/10µl; Santa Cruz Biotechnology, CA) was injected in the rats intrathacally 1, 4, and 14 days after the intraplantar administration of CFA or normal saline.

**Behavioral tests**

The rats were habituated to the apparatus used to measure their behavioral responses for at least 30 min prior to pain evaluation to reduce stress-induced influence. Pain-related parameters were collected prior to intraplantar injection, 4 h, 4 days and 14 days after the intraplantar administration of CFA or saline. These time points were chosen to represent the acute (4 h), subacute (4 days) and chronic (14 days) phases of IP[15, 16]. The experiments were repeated thrice and the average values were obtained for data analysis.

**Thermal withdrawal latency**

Thermal hyperalgesia was assessed using a plantar test (Model 37370; Ugo Basile, Comerio, Italy). Briefly, the rats were placed in a Perspex box on an elevated glass table. A mobile radiant heat source was focused on the plantar surface of the hind paw, and the paw withdrawal latencies were recorded as the time taken by each rat before lifting its hind paw from the increasing thermal stimulus. The cutoff point was set at 20 s to prevent tissue damage.

**Mechanical withdrawal threshold**

Mechanical nociceptive thresholds were determined using a Dynamic Plantar Aesthesiometer (Model 37450; Ugo Basile, Comerio, Italy). Rats were gently placed in the apparatus with a wiremesh floor. A straight metal filament, which exerting an increasing upward force at a constant rate (3 g/s) with a maximum cut-off force of 50 g was placed under the plantar surface of the hind paw. Measurements ended when the paw was withdrawn, and results were expressed as paw withdrawal thresholds in grams.

**Paw pressure**

The mechanical pain threshold in response to pressure was measured using an analgesy meter (Model 37215; Ugo Basile, Comerio, Italy). Rats were lightly restrained, and their left hind paw was placed on a pedestal. Increasing pressure was then applied to the paw through a probe resting on the dorsal surface up to a maximum of 25 g. The force at which the rats struggled to withdraw their paw was considered the nociceptive threshold.

**Weight distribution**

The weight distribution between the rats' hind limbs was determined with the TSE Power Meter System (303550 TSE SYSTEM, Germany). Animals were placed in the apparatus, and their hind paws were
centered on each force plate, which measured the body weight distribution of each hind limb. The average body weight distribution was measured over 10 s in grams. Results were expressed as the percent distribution of weight on the left hind paw using a previously described formula[17].

**Western blotting analysis**

Lumbar spinal cord samples (L4–L5) taken from the rats were then homogenized in lysis buffer. After incubation for 30 min in ice-cold water with constant agitation, the homogenates were centrifuged at 4°C for 20 min at 12,000g. The supernatants were collected and used for Western blot analysis. Total protein concentration was determined using a BCA kit (Pierce, Rockford, IL). Protein samples were dissolved in 4× sample buffer and denatured at 95°C for 5 min. Equivalent amounts of protein (30 µg) were separated using 12% SDS–PAGE and transferred onto a PVDF membrane. The membranes were blocked with 5% fat-free milk solution and incubated overnight at 4°C with anti-HMGB1 or anti-β-actin primary antibodies. The membranes were extensively washed with Tris-buffered saline with Tween 20 and incubated for 1 h with HRP-conjugated IgG secondary antibody (Huamei Chemical Corp., China). Bands were revealed using an ECL kit (Pu fei Chemical Corp., Shanghai, China). Scanning densitometry was used for semiquantitative analysis with a computer-assisted imaging analyzer (Gel Pro Analyzer).

**Real-time reverse transcription polymerase chain reaction (RT-PCR)**

Lumbar spinal cords (L4–L5) samples taken from the rats and preserved in RNAlater (Ambion, Austin, TX) were homogenized using Tissuelyser (MM300; Qiagen, Valencia, CA) with a single 6.5-mm stainless steel ball per sample. Total RNA extraction and RT reaction were performed as previously described[18]. The amounts of target genes, including HMGB1, TNF-α and IL-1β cDNA were normalized against the housekeeping gene β-actin in the corresponding samples. The following primers were used: HMGB1 (NM_002128.4) forward primer: 5′-GGCTGACAAGGCTCGTTATG-3′, HMGB1 reverse primer: 5′-GGCGGTACTCAGAAACAGA-3′; IL-1β (NM_031512.2) forward primer: 5′-GCAACTGTCCCTGAACTCAA-3′, IL-1β reverse primer: 5′-TGTCAGCCTCAAAGAACAGG-3′; TNF-α (NM_012675.2) forward primer: 5′-CTAACTCCCAGAAAAAGCAAGCAA-3′, TNF-α reverse primer: 5′-CCTCGGGCCAGTGTATGAGA-3′; β-actin (NM_031144) forward primer: 5′-CCCTGTTGCGTGTGCTCACCGA-3′, and β-actin reverse primer: 5′-ACAGTGTGGTGACCCCGTC-3′.

Quantitative PCR was carried out under the guidance of the instructions. Using a LightCycler rapid thermal cycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. PCR was performed with 1×LC-DNA Master SYBR Green Mix in a final volume of 20 µl. Real-time detection of SYBR green fluorescence intensity was carried out at the end of each elongation phase. The amplified products were quantified using the LightCycler software package. Data were gathered and analyzed using the comparative Ct method.

**Statistical analysis**
All results were expressed as mean ± SEM. Data from the Western blot analysis and RT-PCR were evaluated using one-way analysis of variance (ANOVA) followed by post hoc Dennett’s test. Data from the behavioral response tests were analyzed using two-way ANOVA to compare differences between groups at different time points, whereas repeated-measures ANOVA was used to evaluate treatment efficiency along different time points within groups. Pos-hoc Bonferroni’s test was used to detect significant differences for both ANOVA designs. $P < 0.05$ was considered statistically significant.

Results

Anti-nociceptive effects of intrathecal injection of anti-HMGB1

Significant behavioral hypersensitivity was observed in rats in response to peripheral administration of CFA. Vehicle and anti-HMGB1 were intrathecally administered in the sham group and IP group, respectively, to determine the effects of the neutralizing antibody of HMGB1 (anti-HMGB1) on the time course of IP. Anti-HMGB1 induced an antinociceptive effect on days 4 and 14 post-injection ($P < 0.05$), but not 4 h ($P > 0.05$) after the intraplantar administration of CFA. Statistical differences between the IP and vehicle groups were not detected ($P > 0.05$). Intrathecal application of anti-HMGB1 had no influence on the behavioral responses of rats in the sham group compared with baseline ($P > 0.05$) (Fig. 1).

Changes in the expression of spinal HMGB1 and the effects of anti-HMGB1

Western blot analysis and RT-PCR were performed to detect the expression of spinal HMGB1 in the different groups. The time-course study showed that HMGB1 expression was not increased at 4 h but markedly enhanced on days 4 and 14 after the intraplantar injection of CFA. To further certify the involvement of up-regulated HMGB1 expression in IP, we intrathecally injected anti-HMGB1 and reevaluated the expression of spinal HMGB1 at different time points. Treatment with anti-HMGB1 significantly decreased, at the protein and mRNA levels, the expression of HMGB1 in rats on days 4 and 14 ($P < 0.05$), but no difference was observed at 4 h ($P > 0.05$). Differences between the vehicle group and IP group were not statistically significant (data not shown: $P > 0.05$) (Fig. 2).

Changes in the expression of spinal proinflammatory cytokines and the effects of anti-HMGB1

To discover the potential mechanisms for the antinociceptive effects of anti-HMGB1, we measured the expression of proinflammatory cytokines, including IL-1β and TNF-α, following anti-HMGB1 injection at the mRNA level. The results showed that both IL-1β and TNF-α were significantly increased in IP rats at each time point (vehicle vs. sham and naive groups, $P < 0.05$) (Fig. 2). Intrathecal injection of anti-HMGB1 significantly down-regulated IL-1β and TNF-α expression on days 4 and 14 ($P < 0.05$), but failed to reduce
the expression at 4 h compared with vehicle treatment \((P > 0.05)\). In contrast, the expressions of IL-1\(\beta\) and TNF-\(\alpha\) in the anti-HMGB1 group were still higher than those in the sham and naive groups \((P > 0.05)\).

**Discussion**

This study has established a novel contribution of spinal HMGB1 in IP. HMGB1 is increased in the spinal cord of rats with peripheral inflammation, and its up-regulated expression correlates with behavioral hypersensitivity at the time course of the subacute (day 4) and chronic phase (day 14) phases of CFA-induced peripheral inflammation, but not at acute phase (4 h). Blocking of spinal HMGB1 could improve IP with down-regulated IL-1\(\beta\) and TNF-\(\alpha\) expression. These findings indicate that HMGB1 as a late proinflammatory cytokine is involved in pathogenesis process of IP.

HMGB1 is known as both secreted protein and a nuclear factor. It can be released into the extracellular space either actively or passively\([19]\). Recent studies have not only shown that extracellular HMGB1 acting as a proinflammatory cytokine plays an important role in the pathogenesis of various inflammatory conditions\([20]\). To clarify the involvement of HMGB1 in IP-related behavior, we used a neutralizing antibody against HMGB1, which has proven effects in arthritis\([21]\), necrosis\([22]\), neuropathic pain\([13]\), and bone cancer pain\([23]\). We found that anti-HMGB1 could attenuate IP with down-regulated IL-1\(\beta\) and TNF-\(\alpha\) expression at the subacute and chronic phases of inflammation. Up-regulated expressions of the spinal proinflammatory cytokines IL-1\(\beta\) and TNF-\(\alpha\) were detected throughout the phases of CFA-induced peripheral inflammation. We speculated that the up-regulated expressions of IL-1\(\beta\) and TNF-\(\alpha\) contributed to the behavioral hypersensitivity of the rats at the acute phase of IP.

The activation of spinal neurons and glia via nociceptive information plays a remarkable role in the chronic pain states by releasing proinflammatory cytokines, including HMGB1\([24, 25]\). Intriguingly, we did not detect the up-regulated expression of HMGB1 at the protein and mRNA levels in the acute phase of IP. Moreover, the administration of anti-HMGB1 failed to reverse pain hypersensitivity at the same time point. These findings indicate that HMGB1 acting as a late proinflammatory mediator is involved in the pain pathogenesis process. The results for the subacute and chronic phases in our studies confirmed our hypothesis.

Extracellular HMGB1 binds to its receptors, such as RAGE, TLR-2 and TLR-4. RAGE has been reported to be present in the spinal nerve, and the interaction of HMGB1 with RAGE not only induces secretion of proinflammatory cytokines\([23]\) but also can activate the receptor to produce behavioral hyperalgesia\([26]\). In addition, high levels of TLR-2 and TLR-4 expression in the spinal cord and targeting TLRs have been indicated as critical mediators of chronic pain\([27]\). TLRs signal through myeloid differentiation factor-88-independent pathways, which activate the NF-\(\kappa\)B family and subsequently result in the transcription of genes of proinflammatory cytokines\([28]\). These findings indicate that HMGB1 contributes to inflammatory hypersensitivity via interactions with its receptors. After binding the receptors, HMGB1 can trigger its downstream cascade responses, followed by up-regulation of proinflammatory mediators, such as nitric oxide synthetase, cytokines, and chemokines\([29]\).
This study has demonstrated that the expressions of IL-1\(\beta\) and TNF-\(\alpha\) in the spinal cord correlate with the expression of HMGB1 during the IP state. The findings suggest that, at the acute phase, increased IL-1\(\beta\) and TNF-\(\alpha\) may not only contribute to the early inflammatory hypersensitivity but also activate neurons and glial cells resulting in the secretion of HMGB1. HMGB1, together with cytokines, could induce a positive feedback loop on the cascading inflammatory process during the subacute and chronic phases of pain, which probably aggravates the spinal neuroimmune interactions; ultimately potentially triggering prolonged IP.

In conclusion, peripheral inflammation results in up-regulated expression and release of HMGB1 in the spinal cord. Anti-HMGB1 treatment can alleviate IP hypersensitivity induced by intraplantar administration of CFA with down-regulated expression of IL-1\(\beta\) and TNF-\(\alpha\).

**Conclusion**

HMGB1 is thus an important component of the progression of pain hypersensitivity after peripheral inflammation, and blocking HMGB-1 signaling may be a potential therapeutic approach for the treatment of IP.

**Declarations**

**Ethics approval**

Medical Ethics Committee of Lianyungang Maternal and Child Health Hospital approved all the protocols of the study LW2022010.

**Approval for animal experiments**

Medical Ethics Committee of Lianyungang Maternal and Child Health Hospital approved all the protocols of the study LW2022010. We confirm that all experiments were performed in accordance with relevant named guidelines and regulations and authors complied with the ARRIVE guidelines.

**Consent to publish**

The authors declare that all work described here has not been published before and that its publication has been approved by all co-authors.

**Availability of data and materials**

All data generated or analysed during this study are included in this published article.

**Competing interests**

The authors declare that they have no competing interests.
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Author contributions

YW, JF, JC and LZ, designed the study and discussed the outcome of experiments and wrote the paper. JC, LZ, YM and PT performed Western blot and behavioral experiments. GZ performed Western blot assay.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

References


Figures
Figure 1

Anti-nociceptive effects of intrathecal injection of anti-HMGB1. (A-D) Rats subjected to intraplantar administration of CFA displayed significant nociceptive hypersensitivity compared with sham rats. Intrathecal injection of anti-HMGB1 produced anti-nociceptive effects on days 4 and 14 post-injection but not 4 h after CFA was injected compared with CFA and vehicle treatments. Results are expressed as mean
± SEM, n=10, #P<0.05, ##P<0.01 vs. sham and sham + anti-HMGB1 groups; *P<0.05, **P<0.01 vs. CFA and CFA + vehicle groups.

Figure 2

(A, B) Western blot analysis revealed that HMGB1 protein expression in the L4-L6 spinal cord increased on days 4 and 14 after the intraplantar injection of CFA compared with that in sham and normal rats (P<
0.05, n = 4). Anti-HMGB1 decreased HMGB1 overexpression induced by the CFA. (C-E) Real-time RT-PCR showed that HMGB1 mRNA expression was up-regulated on days 4 and 14, whereas the expressions of TNF-α and IL-1β mRNA were up-regulated at 4 h and on days 4 and 14 post-injection compared with those in sham and normal rats (P < 0.05, n = 4). Anti-HMGB1 down-regulated the cytokine mRNA on days 4 and 14 compared with CFA treatment. Results are expressed as mean ± SEM, n = 4, #P < 0.05, ##P < 0.01 vs. sham and normal groups; *P < 0.05, **P < 0.01 vs. CFA group.