

Original Article

Spinal IP-10 participates in antinociception of electroacupuncture in osteoarthritis pain

Shisheng Li, Yaochi Wu, Junfeng Zhang, Shenghong Zhang, Jing Liu

Department of Acupuncture, Tuina and Traumatology, The Sixth People's Hospital Affiliated to Shanghai Jiaotong University, Shanghai 200233, China

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Abstract: This study aimed to investigate whether electroacupuncture (EA) could exert analgesic effect by up-regulation of opioid peptide expression and suppression of microglia activation in spinal cord of rat with osteoarthritis (OA), and explore the role of interferon gamma-induced protein 10 (IP-10)/CXC chemokine receptor 3 (CXCR3) in this process. Following establishment of OA through transecting the anterior and posterior cruciate ligaments and removing the meniscus, the rats were treated with EA at GB30 (Huantiao) and GB34 (Yanglingquan). After 4 days, the mechanical and thermal nociceptive thresholds were determined. The microglia activation was assayed through determination of Iba-1 expression. The levels of IP-10, CXCR3, β -endorphin (END), Met-enkephalin (ENK) and Dynorphins (DYN) were measured by qRT-PCR and western blotting. To investigate the role of IP-10 in the antinociception of EA, the rats were administrated with recombinant IP-10 after EA stimulation, and the nociceptive thresholds, microglia activation and opioid peptide production were measured as described above. Surgery induced OA reduced the mechanical and thermal nociceptive thresholds in rats, and promoted microglia activation and the expression of IP-10, CXCR3, END, ENK and DYN. EA treatment considerably increased the nociceptive thresholds, attenuated microglia activation and the expression of IP-10 and CXCR3, and upregulated the opioid peptide levels, which were reversed by recombinant IP-10. In conclusion, EA could enhance the nociceptive thresholds in rats with OA through inhibition of microglia activation and upregulation of opioid peptide, which was related to the altered expression of IP-10.

Keywords: Osteoarthritis pain, electroacupuncture, IP-10, microglia, opioid peptide

Introduction

Osteoarthritis (OA) is the most common type of arthritis, involving the articular cartilage, adjacent bones, meniscus, ligaments, muscles and joints, almost the whole joint, and the clinical features of OA are mainly joint pain and loss of joint function [1]. The primary symptom of OA is pain, which drives individuals to seek medical attention and contributes to limited joint function [2]. OA pain has always been considered to be a nociceptive pain caused by local tissue injury. However, lots of basic research, animal experiment and clinical studies have suggested that both nociceptive and neuropathic pain mechanisms are involved in the OA pain, and with the progress of this disease, OA pain will transform from nociceptive pain to neuropathic pain [3, 4].

Recent studies on the mechanism of OA-associated pain have demonstrated that peripher-

al and central sensitization contributes to OA pain [5]. The occurrence of central sensitization involves a variety of neural and glial processes, in which spinal glial cells activation induced by inflammation or injury may be one of the necessary conditions for the initiation and maintenance of spinal cord sensitization. In the MIA-induced OA model, microglia and astrocytes were activated sequentially, and nimesulide, a COX inhibitor, or minocycline attenuated pain behavior, and the activation of microglia and astrocyte in the ipsilateral spinal cord, which indicated that spinal glia cells were involved in OA-induced pain [6]. In addition, the protein levels of multiple pro-inflammatory cytokines and chemokines such as IL-1, CCL5, TNF- α and vascular endothelial growth factor (VEGF) were significantly increased in the lumbar spinal dorsal horn at 5 weeks after MIA injection [7]. A recent study found CCL2 and its receptor, CCR2, significantly increased in L3-5 DRG neurons at 8

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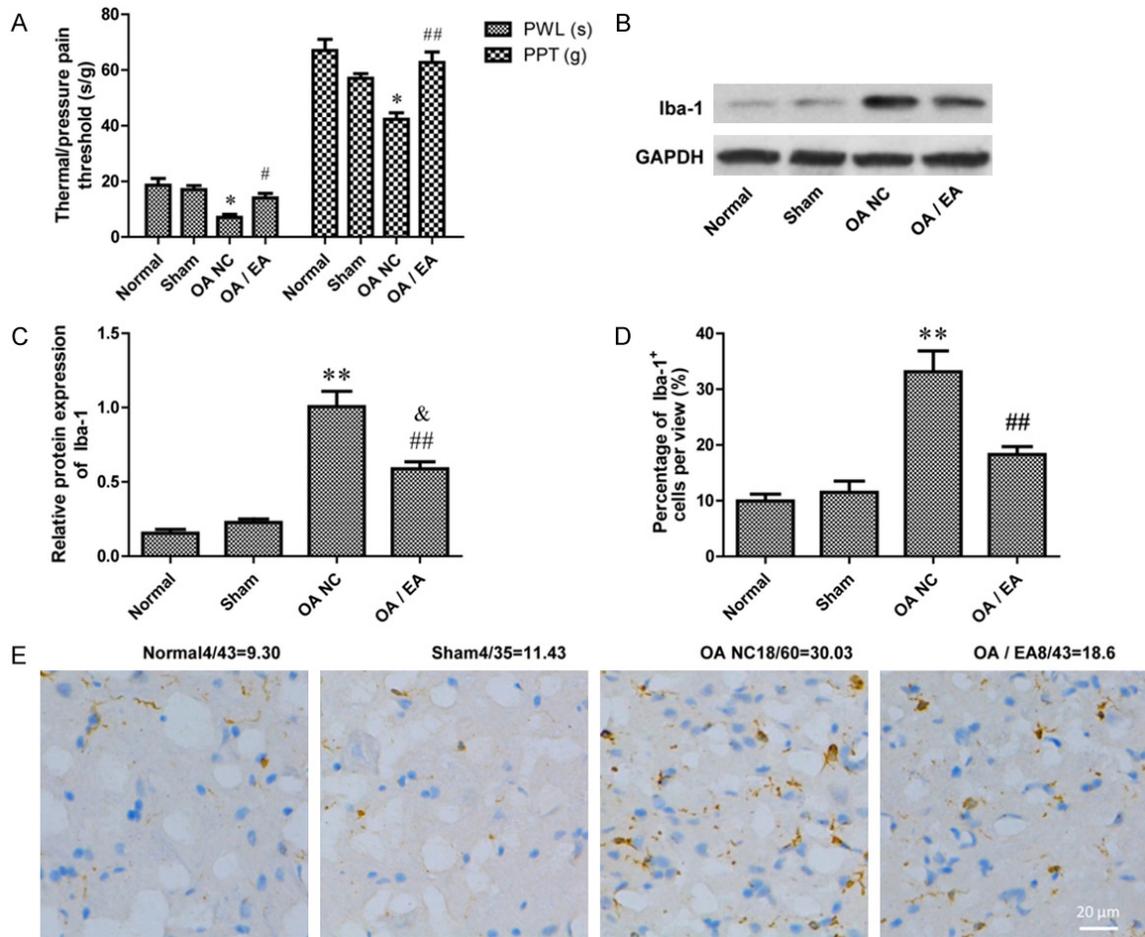


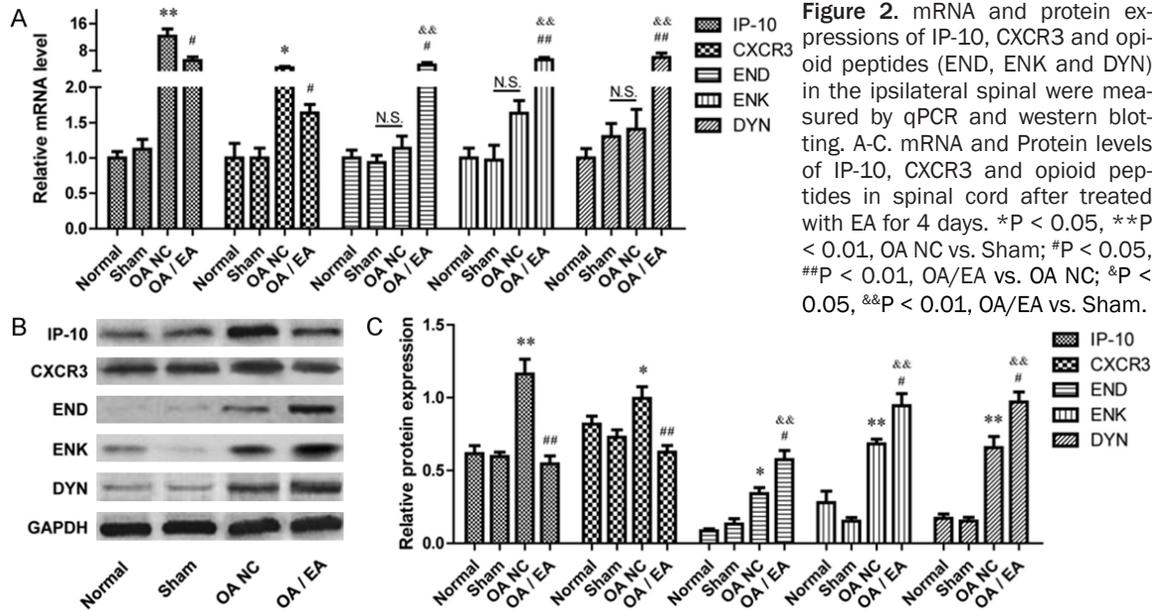
Figure 1. Thermal and pressure pain thresholds and microglia activation in spinal cord of OA rats were altered by electroacupuncture (EA) treatment. A. Thermal (paw withdrawal latency, PWL) and pressure (paw pressure threshold, PPT) pain thresholds of OA rats after treated with EA. B. Iba-1 protein level in spinal cord measured by western blotting. C. Gray analysis based on three repeated western blots. D. Number of Iba-1 positive (Iba-1⁺) cells per view in spinal cord based on immunohistochemical staining. E. Representative results of immunohistochemical staining of Iba-1 in spinal cord. *P < 0.05, **P < 0.01, OA NC vs. Sham; #P < 0.05, ##P < 0.01, OA/EA vs. OA NC; &P < 0.05, &&P < 0.01, OA/EA vs. Sham.

weeks and returned to base levels at 16 weeks post-destabilization of the medial meniscus surgery, which was accompanied by the movement-provoked pain behaviors, and the pain could be reversed by administration of a CCR2 antagonist [8]. These data suggest that chemokines and their receptors may participate in the development and maintenance of OA pain. In addition to microglia and chemokines, opioid peptides are also involved in the OA induced pain. In patients with OA, END and ENK were mostly expressed by macrophages/monocytes, lymphocytes and plasma cells, and μ and δ opioid receptors (ORs) were found on nerve fibers and immune cells, and were co-expressed with neuronal markers calcitonin gene-related peptide. These findings demonstrated that with

prolonged and enhanced inflammation, the immune and peripheral nervous systems up-regulate sensory nerves expressing ORs and their ligands to counterbalance pain and inflammation [9].

At present, there is no satisfactory treatment for OA, and the current standard of care is to manage and alleviate symptoms. Despite the use of various conservative and interventional treatment of pain, the efficacy is not satisfactory, and most OA patients still experience pain. Electroacupuncture (EA), an acupuncture therapy, has exhibited analgesic effects on different types of acute, persistent inflammatory and neuropathic pain [10]. Several processes have been proposed to explain the analgesic effects

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of EA, such as down-regulation of pro-inflammatory cytokines [11], stimulation of the release of neurotransmitters or neuromodulators including opioid peptides, serotonin and noradrenaline [12], activation of pain inhibitory systems [13], involving opioid, adrenergic, dopaminergic, serotonergic, and cholinergic receptors [14, 15], and inhibition of spinal glial activation [16].

The effects of EA on OA induced pain and the underlying mechanism have not been investigated so far. IP-10 is a small cytokine secreted by several cell types include monocytes, endothelial cells and fibroblasts, and could trigger early microglial activation in the cuprizone model and control inflammatory pain via inducing opioid peptide containing CXCR3+ macrophages aggregation [17, 18]. In this work, we attempted to explore whether EA treatment could ameliorate OA induced allodynia in rats, the role of spinal microglial activation and opioid peptide release in this process and whether IP-10 contributes to the analgesic effects of EA in OA pain.

Results

EA treatment increased the nociceptive thresholds of rats with OA and attenuated spinal microglia activation

To evaluate the antinociception of EA in OA pain, the thermal and mechanical pain threshold of rats from different groups were deter-

mined after EA treatment for 4 days. We found that EA treatment effectively improved the mechanical allodynia and thermal hyperalgesia induced by OA surgery (**Figure 1A**). To assess the participation of the spinal microglia in the generation of mechanical and thermal hypersensitivity in OA rats, we measured the expression of Iba-1, a marker of activated microglia, using western blotting and immunohistochemical methods. The results showed surgery induced OA significantly increased the protein level of Iba-1 and numbers of Iba-1 positive cells in spinal cord (**Figure 1B-E**), which might be attributed to the activation and aggregation of microglia in response to injury or stress. However, EA treatment markedly decreased OA induced Iba-1 production in spinal cord (**Figure 1B-E**). In conclusion, the inhibition of microglia activation by EA may contribute to the antinociception of EA in OA pain.

EA treatment reduced the expression of IP-10 and CXCR3 and increased the expression of opioid peptides in the spinal cord

To explore the role of IP-10/CXCR3 in the generation of OA pain and the antinociception of EA in OA, the expression of IP-10 and CXCR3 in the spinal cord were measured by western blotting. We found surgery induced OA observably promote the transcription and translation of IP-10 and CXCR3 in spinal cord (**Figure 2**), which indicated that the interaction between IP-10 and CXCR3 may play vital role in the develop-

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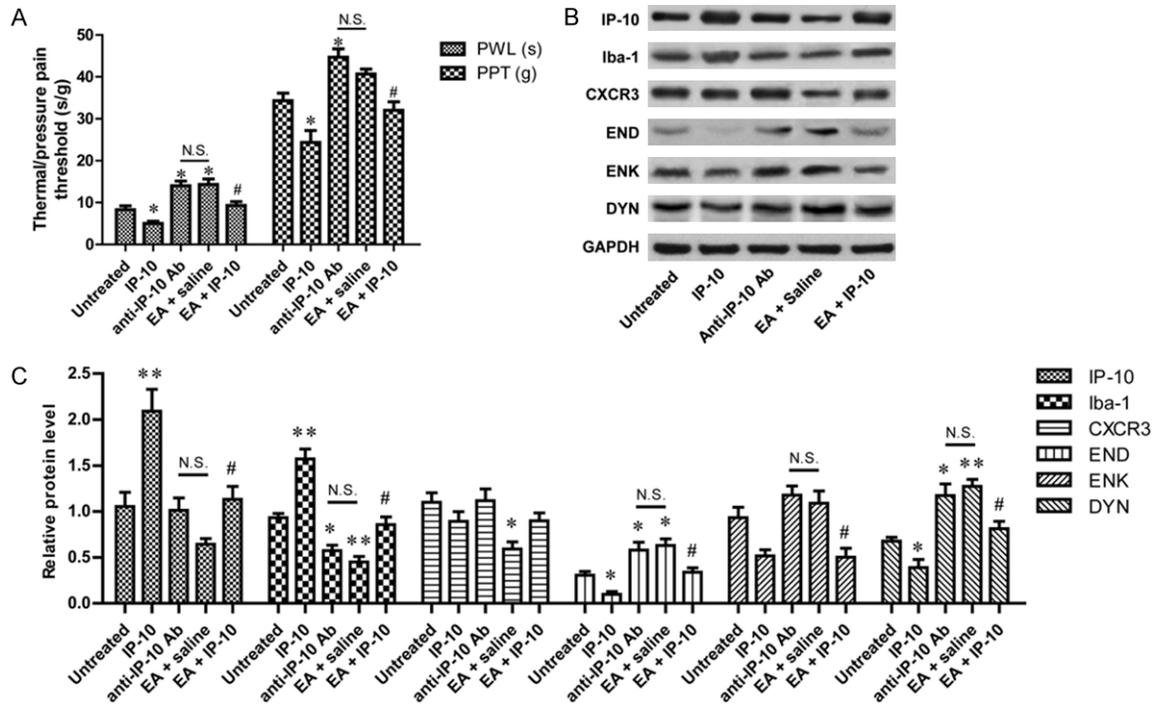


Figure 3. Recombinant IP-10 reversed the antinociceptive effect of EA in treating osteoarthritis pain and altered expression of Iba-1, CXCR3, and opioid peptides END, ENK and DYN. (A) EA-induced increase of thermal and pressure pain thresholds were eliminated by treating with recombinant IP-10. (B, C) Iba-1, IP-10, CXCR3, END, ENK and DYN protein levels were altered by recombinant IP-10, where the protein levels were measured with western blotting (B) and quantified using gray analysis (C). * $P < 0.05$, ** $P < 0.01$, compared with the untreated group; # $P < 0.05$, ## $P < 0.01$, EA+IP-10 group vs. EA+ Saline group.

ment of allodynia in rats with OA. EA treatment could effectively attenuate the increased expression of IP-10 and CXCR3. Accumulating studies have confirmed that endogenous opioid peptides including END, ENK and DYN could contribute to the anti-allodynic effect of acupuncture, and acupuncture could promote the expression of these opioid peptides. Consistent with previous studies, in this work we found the protein expression levels of END, ENK and DYN in spinal cord were significantly increased after the occurrence of OA, which were further improved by the EA treatment (Figure 2).

The improvement effect of EA on allodynia was reversed by intrathecal injection of recombinant IP-10

To confirm the role of IP-10 in the antinociception of EA in OA pain, rats with OA were subsequently intrathecally injected with recombinant IP-10 or anti-IP-10 Ab, and the mechanical and thermal nociceptive thresholds, microglia activation and the protein expression of END, ENK and DYN were determined. The rats adminis-

trated with recombinant IP-10 showed significantly reduced nociceptive thresholds, increased microglia activation and decreased levels of opioid peptides (Figure 3). In contrast, the nociceptive thresholds and protein levels of END, ENK and DYN were remarkably increased in rats received anti-IP-10 Ab, and has not significant difference with that in rats received EA treatment alone (Figure 3). In addition, the microglia activation was also considerably ameliorated by anti-IP-10 Ab injection. Moreover, the influence of EA treatment on nociceptive thresholds, microglia activation and the protein expression of opioid peptides were abolished by the injection of recombinant IP-10 (Figure 3). All these findings demonstrated the vital role of IP-10 in the antinociception of EA in OA pain, and the mechanism might be related to the reduced microglia activation and increased levels of opioid peptides.

Discussion

Acupuncture has been used for thousands of years in Eastern countries to treat various mal-

adies, especially pain and is now recognized as a potential therapy in Western countries. Acupuncture involves the insertion and manipulation of microneedles into specific body sites, namely acupoints, on the meridian [16]. Due to the heavy workload of manual acupuncture, a modified acupuncture technique utilizing electrical stimulation was invented to apply this therapy to more patients, so called electroacupuncture (EA). Accumulating evidence suggests that EA are effective for the management and treatment of different types of acute pain, persistent inflammatory pain and neuropathic pain [19, 20]. Despite its widespread use, the underlying mechanisms for the analgesic effects of EA are still incompletely understood, and need further well-controlled randomized studies. Several processes have been proposed to explain EA induced analgesia, in which, the inhibitory effect of EA on microglia activation received the most extensive attentions.

For a long time, the research on EA analgesia is mainly focused on the effect of EA on neural network of neurons and various kinds of nerve active substances released by neurons. Recent studies have indicated that the activation of spinal cord glial cells is closely related to the occurrence and development of pain and EA has a certain regulating effect on the activation of glial cells. Thus, the research emphasis of the studies on the mechanism of EA analgesia has turned from neurons to glial cells or glial cells-neuronal network. It is well known that glial cells in resting state are not involved in the transmission and regulation of pain information, and only activated glial cells contribute to the amplification of pain through releasing of various neural and glial active substances such as proinflammatory cytokines, adenosine triphosphate (ATP). The glial cells in spinal cord associated with pain are mainly astrocytes and microglia, which play different roles in the generation and maintenance of pathological pain. Microglia play a major role in the initiation of pathological pain, and mostly astrocytes participate in the maintenance of pathological pain.

Microglia as the primary immune cells in the central nervous system (CNS) [21], shares many of the same functional and phenotypic features as macrophages, participate in innate immune responses, and become the first line of defense against pathogen invasion in CNS

even before the penetration of peripheral white blood cells. Spinal glial activation has been extensively observed in multiple pain models and patients with pathological pain. It was found that spinal microglia activation began to appear at 1 d after injection of complete Freund's adjuvant (CFA) into the ankle joint cavity of rats, peaked at 3 d, and maintained at a high level till the 10th day [22]. In the pathological pain models of spare nerve injury (SNI), chronic constriction injury (CCI) and spinal nerve ligation (SNL), the expression of CD11b, a marker of microglia activation, in the ipsilateral spinal dorsal horn and ventral horn of the patients began to increase after 3 days of nerve injury, reached the peak after 7 days, and began to decline after 14 days [23]. Consistent with previous studies, in this work we found spinal microglia activation was significantly enhanced at 4 d after the OA surgery, suggesting microglia activation might contributed to the OA induced thermal hyperalgesia and mechanical allodynia.

In the present study, we demonstrated that EA treatment could effectively suppress OA induced microglia activation in spinal cord. The inhibition effect of EA on microglia activation has been widely reported. A research by Gim et al. found that repeated EA stimulation for 53 days significantly inhibited inflammation and microglia activation in spinal cord of rats with neuropathic pain induced by neuropathic surgery [10]. Sun Shan et al. demonstrated EA stimulation of ipsilateral "Huantiao" (GB30) and "Yanglingquan" (GB34) acupoints significantly suppressed nociceptive behavioral hypersensitivity and spinal microglial activation induced by unilateral intraarticular injection of CFA [24]. However, the molecular mechanism of these observed phenomena is not clear yet. It was postulated that EA might affect glial cells directly or indirectly via opioid receptors [10]. Here, we demonstrated that the interaction of IP-10 and its receptor CXCR3 might contribute to the inhibition effect of EA on OA induced microglia activation. The role of chemokines and chemokine receptors in the generation, transmission and modulation of pain has been extensively studied. It was found that the expression of CCL2 and its receptor CCR2, mainly expressed on the surface of peripheral monocytes/macrophages and microglia, was both increased in peripheral nerve injury [25,

26]. Both intraspinal injection and subcutaneous injection of CCL2 could induce pain and microglia activation in experimental animals [27]. It was showed that total CCR2 knock-out mice did not develop microglial activation or mechanical allodynia after peripheral nerve injury induced by a partial ligation on the sciatic nerve, and intrathecal injection of CCL2 could not cause microglia activation in CCR2-deficient mice [28]. Hypodermic injection of CCL2 neutralizing antibody or CCR2 antagonist prevented could reduce neuropathic pain and inhibiting microglia activation. Furthermore, CCL2 can also attract mononuclear cells, including macrophages and microglia to the area of inflammation or injury [29]. Cathepsin S-Fractalkine-CX3CR1 is another important pain pathway. Fractalkine can be cleaved by Cathepsin S to produce soluble Fractalkine, which may induce the migration and activation of microglial cells, improve the pathological response of nerve, and cause neuropathic pain [30]. Fractalkine is expressed in DRG and dorsal horn of spinal cord in normal condition, and is also expressed in astrocytes after nerve injury [31]. CX3CR1 is expressed by a variety of cell types, including astrocytes and microglia, and its expression is up-regulated in pathological pain. Cathepsin S is released by activated microglia, and intrathecal injection of Cathepsin S induced allodynia and ectopic pain immediately. Intrathecal injection of Fractalkine in rats could cause abnormal pain, while intrathecal injection of the neutralizing antibodies for Fractalkine or CX3CR1 could reduce the pain of animals caused by nerve damage and the phosphorylation of P38 [30, 32]. It was also been found intradermal injection of CXCL8 induced allodynia in rats, which could be efficiently attenuated by CXCL8 antibody [33]. Furthermore, in the Carrageenan pain model, the pain sensitivity could also be eliminated by specific anti CXCL8 serum, suggesting CXCL8-CX3CR1 pathway might also be involved in the modulation of pain [34]. CCL21, also known as secondary lymphoid-tissue chemokine (SLC), mainly distributed in peripheral immune organs or tissues, has chemotaxis effect on a variety of immune cells. Injection of CCL21 into rat ventrolateral nucleus of thalamus induced microglia activation and pain sensitization, and injection of CCL21 neutralizing antibody into the thalamus could inhibit the microglia activation and the emergence of pain sensitiza-

tion, indicating CCL21 is also one of the molecules involved in the regulation of pain [35].

CXCR3 belongs to the CXC chemokine receptor family, and mainly expressed in T cells, natural killer cells, and some epithelial cells and endothelial cells in periphery [36]. In the CNS, *in situ* hybridization experiments showed that CXCR3 was expressed in astrocytes and microglia [37]. However, some other researchers argued that CXCR3 was mainly expressed in neuron and microglia. Aggregation of a large number of glial cells was found on the edge of injured region in multiple sclerosis, which was accompanied by the increased expression of CXCR3 and IP-10. In normal wild-type mice, microglia migrated to the lesions in 3 days after brain injury, while microglia aggregation was inhibited in CXCR3 knockout mice [38]. In addition, recent studies have found that CXCR3 inhibitors have an inhibitory effect on gliomas [39]. These dates suggested that CXCR3 might play a role in the CNS through chemotaxis of glial cells. The involvement of CXCR3 in pain regulation of microglia has not been reported. In this work, we found the expression of IP-10 and CXCR3 was significantly improved in rats with OA, and has a significant positive correlation with microglia activation, suggesting IP-10/CXCR3 might be involved in microglia activation under OA condition. It was demonstrated early microglia activation was significantly reduced in IP-10-deficient mice treated with in short-term cuprizone and *in vitro*, recombinant IP-10 induced migration and a proinflammatory phenotype in cultured microglia, without affecting their phagocytic activity or proliferation [18]. Moreover, repeated intrathecal injection of CXCR3 antagonist AMG487 could significantly inhibit the spinal cord microglial activation and pain in rats with bone cancer [40]. Thus, we speculated that the expression of IP-10 and CXCR3 was downregulated by EA treatment, which led to the reduction of microglia activation and ultimate improvement of pain threshold in OA rats.

It is well documented that endogenous opioid peptides in the CNS are involved in mediating analgesic effects of EA [12, 41]. However, the underlying mechanisms are not fully established. Chemokines and opioids are both key regulators of immune, inflammatory and neuronal responses in peripheral and central pain pathways. In addition to inducing peripheral and central algogenesis, chemokines might

also contribute to antinociceptive effects by attracting opioid-containing leukocytes (such as macrophages and T lymphocytes) to migrate into the damaged tissue, release opioid peptides, which induce antinociception by binding to opioid receptors on peripheral nociceptive neurons [42]. However, studies investigating the effects of chemokines on the contents of opioid peptides in the CNS are rare. In this work we found intrathecal injection of recombinant IP-10 significantly decreased the expression of opioid peptides, including END, ENK and DYN in spinal cord, which was in line with the reduced pain threshold. Further studies are needed to clarify the molecular mechanisms for this phenomenon.

Conclusion

These data suggest that EA treatment decreases IP-10 expression in spinal cord, which ameliorates OA induced allodynia in rats through inhibition of spinal microglia activation and increase of opioid peptide contents. Our data suggest that IP-10 is a key antinociceptive mediator in EA analgesia, and may serve as a promising target for therapy of pain.

Methods and materials

Experimental animals

Male Sprague-Dawley rats, weighting 200-250 g, 7-weeks old, supplied by the Shanghai SLAC laboratory animal Co., Ltd. (Shanghai, China) were housed in group cages (3-4 per cage) and kept under controlled conditions ($22 \pm 0.5^\circ\text{C}$, relative humidity 40-60%, 12-h light/dark cycle, food and water ad libitum). All rats were allowed to acclimate in their cages for 1 week prior to any experiments. All the animal procedures were approved by the Institutional Animal Care and Use Committee of The Sixth People's Hospital Affiliated to Shanghai Jiaotong University and were performed according to the guidelines of the International Association for the Study of Pain [43].

Induction of OA

OA in rats was surgically induced as described previously in the literature [44]. Briefly, the rats were anesthetized with 2% Halotane, and the hair over the medial aspect of the left knee joint was shaved and the skin was disinfected with 70% alcohol. A medial parapatellar inci-

sion was made to expose the knee joint. Then the medial collateral ligament was sharply divided. Then a medial parapatellar arthrotomy was carried out and followed by patella dislocated. With the knee flexed, the anterior/posterior cruciate ligament was transected and the knee joint was then dislocated to excise the medial meniscus. Normal saline was used to irrigate the joint. The capsule and the synovium were closed together with a 4.0 interrupted Vicryl and the skin was closed with 4.0 silk suture. The joint capsule of rats received sham procedure was opened as described. After dislocation of patella, the ligaments and menisci were kept intact. After patella reduction and knee irrigation with saline, the joint capsule and the incision was closed gradually. Post-operative, the rats were given intramuscular injections of penicillin 1 ml (4×10^7 U/L) for 1 time for infection prevention. Tramadol (10 mg/kg) was used to control pain after the rats recover.

Acupuncture treatment

For EA stimulation, the rats were fixed in a specially designed wooden restrainer with the head, hind legs and tail protruding. When the rats calmed down for 20 min, a pair of stainless steel acupuncture needles (0.34 mm in diameter) were inserted (5 mm in depth) into the left (ipsilateral) "Huantiao" (GB30, located the lateral 1/3 and medial 2/3 of the distance between the sacral hiatus and the greater trochanter of femur), and "Yanglingquan" (GB34, located in the depression anterior and inferior to the fibula capitulum) acupoints. "Huantiao" and "Yanglingquan" acupoints are commonly used in clinical treatment of rheumatoid arthritis, so these acupoints were used as the stimulating points of EA in this work. The acupuncture needles were connected with the output terminals of the H.A.N.S. Acupoint Stimulator (LH-202H, Huawei Co., Ltd., Beijing, China). Treatment parameters: square wave current output (pulse width: 0.2 ms); intensities ranging from 1 to 2 to 3 mA (each intensity for 10 min, totaling 30 min); at a 100 Hz and 2 Hz alternating frequencies (automatically shifting between 100 Hz and 2 Hz stimulation for 5 s each). EA treatment was performed once at 30 minutes preoperative and once a day for 4 days postoperative. For sham EA treatment, the needles were inserted into the acupoints but without electrical stimulation.

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Intrathecal surgery and drug injection

Intrathecal intubation was performed under chloral hydrate (0.35-0.40 g/kg, i.p.) anesthesia, and the absent corneal reflex was regarded as the index of successful anesthesia. In the prone position, a PE-10 tube was inserted through the gap between the L4 and L5 vertebrae and extended to the subarachnoid space of the lumbar enlargement (L4 and L5 segments), and the presence of cerebrospinal fluid outflow was regarded as a symbol of successful intubation. The PE-10 tube was filled with sterile NS, and the outer end was plugged. Postoperatively, the animals were subcutaneously injected with penicillin G potassium (10^5 U/d) once a day to the infection. The animals were allowed to recover for 3-4 days, and any animals with symptoms of spinal cord injury were excluded from the experiment. Recombinant IP-10 (400-33; Peprotech, RH, USA) and anti-IP-10 Ab (500-P290; Peprotech, RH, USA) were freshly dissolved daily in NS. Drug or vehicle (NS) was injected over a period of 1 min via the PE-10 tube at a volume of 10 μ l, followed by 5 μ l NS for flushing.

Experimental procedures

Two sets of experiments were performed: (1) EA and (2) EA plus recombinant IP-10 and anti-IP-10 Ab.

In Experiment 1, rats were randomly divided into the following groups (n = 10): Normal group, rats only received sham EA treatment; Sham group, rats received sham surgery and sham EA treatment; Model control (OA NC), rats received OA surgery and sham EA treatment; EA treatment group (OA/EA), rats received OA surgery and EA treatment.

In Experiment 2, rats were divided into the following groups (n = 10): Untreated group, rats only received OA surgery; Recombinant IP-10 treatment group (OA/recombinant IP-10), rats were intrathecally injected with 20 ng of recombinant IP-10 at 1 h post OA surgery; Anti-IP-10 Ab treatment group (OA/anti-IP-10 Ab), rats were intrathecally injected with 100 ng of anti-IP-10 Ab at 1 h post OA surgery; EA plus saline treatment group (OA/EA), rats with OA were intrathecally injected with saline at 30 min post EA treatment; EA plus recombinant IP-10 treatment group (OA/EA+ recombinant IP-10), rats with OA were intrathecally injected with 20 ng

of recombinant IP-10 at 30 min post EA treatment. After determination of nociceptive thresholds, rats were sacrificed for immunohistochemistry, qRT-PCR and Western blot analysis on day 5.

Behavioral testing

Hargreaves' test for thermal hyperalgesia: Rats were tested for hindpaw thermal hyperalgesia by a previously described method [45]. Animals were placed individually into a clear plastic chamber on the glass surface of the Paw Thermal Stimulator System (UCSD, San Diego). After acclimation to the environment, a radiant heat stimulus was applied from underneath the glass floor with a high intensity projector lamp bulb. The heat stimulus was directed onto the plantar surface of each hind paw, and the PWL to the nearest 0.1 s was automatically determined. The intensity of the thermal stimulus was adjusted to derive an average baseline PWL of approximately 8-12 s in naive animals. A 20-s cut-off was used to prevent tissue damage.

von Frey test for mechanical allodynia: Mechanical allodynia of the hindpaw was accessed by measuring the withdrawal threshold in response to a series of calibrated von Frey filaments (Stoelting, IL, USA). Animals were placed individually into Plexiglas chambers with customized platform of 5 mm in thickness, which contains 1.5 mm diameter holes throughout the entire area of the platform. Before the test, rats were allowed to adapt to the test chambers for 30 minutes, and the temperature was controlled at $20 \pm 2^\circ\text{C}$. Then, a series of 9 calibrated von Frey hairs were applied to the central region of the plantar surface of one hindpaw in ascending order (1, 1.4, 2, 4, 6, 8, 10, 15, and 26 g). A particular hair was applied until buckling of the hair occurred. The stimulation was maintained for approximately 2 s, 5 times in a row, and the interval was 15 s. A withdrawal response was considered valid only if the hindpaw was completely removed from the customized platform. The hindpaw withdrawal threshold (PWT) was defined as the lowest hair force in grams that produced at least three withdrawal responses in five tests.

Immunohistochemistry

The rats were perfused with 150 ml of 37°C normal saline (NS), and fixed with 400 ml of 4% paraformaldehyde in 100 mM phosphate buf-

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fer (4°C) under deep anesthesia by ethyl carbamate (2.5%, 1.5 g/kg, i.p.). The total perfusion time was about 40-60 min. At the end of perfusion, the L1-S2 segments of spinal cord were removed immediately, post-fixed in the same fixative for 4 h at 4 °C, and immersed from 10-30% sucrose solution for 24-48 h at 4°C for cryoprotection. Tissues were sectioned at 40 µm on a freezing microtome (Leica, Nussloch, Germany) under OTC (Miles Inc., Elkhart, IN) protection. Six to eight sections for each rat were randomly selected. After rinsed with 0.01 M PBS for three times, the sections were blocked with 10% normal donkey serum in 0.01 M PBS with 0.3% Triton-X-100 over night at 4°C. Then the sections were incubated with mouse anti-Iba-1 (1:1000, ab15690, Abcam) primary antibody at 4°C for 24 h. The sections were then washed with 0.01 M PBS for 3 times, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rat IgG (1:100, ab102181, Abcam) for 2 h at 4°C. After washed with PBS in dark and mounted with fluorescent mounting medium (0.01 M PBS: glycerinum, 1:1), the sections were examined under fluorescence microscope (Leica, Germany). Image acquisition was accomplished using Spot digital camera (CTS, USA) and corresponding software (Spot advance). Cell counting was performed by a blinded investigator using NIH Image J software (Bethesda, MD, USA).

Western blotting

The rats were executed after anesthetized by intraperitoneal injection of ethyl carbamate (2.5%, 1.5 g/kg). The L4-S5 segments of spinal cord were isolated immediately in ice bath and divided into two parts along the ventral midline. The left part (OA side) was weighed and frozen at 70°C for further processing. Frozen spinal cords were homogenized on ice in RIPA buffer (80 mg/ml) supplemented with proteases inhibitor cocktails (Sigma-Aldrich, France). Protein concentration was determined using a Bio-Rad protein assay (Bio-Rad, Paris, France). Then, equal amounts of proteins (30 µg) was mixed with equal volume of 2× loading buffer, heated at 100°C for 2-3 min and separated by SDS-PAGE gel (10% acrylamide), and subsequently transferred (Trans-Blot SD; Bio-Rad) onto a PVDF membrane (Bio-Rad). After blocking with TTBS containing 20% dried skimmed milk at room temperature for 2 h, the membranes were then incubated with primary antibodies (anti-

Iba-1, 1:1000, ab15690, Abcam; anti-IP-10, 1:5000, ab7206, Abcam; anti-CXCR3, 1:1000, 49801, Novus; anti-β-END, 1:1000, sc-18264, Santa Cruz Biotechnology; anti-ENK, 1:200, orb6690, Biorbyt; anti-PDYN, LS-C116726, LS-Bio and anti-GAPDH, 1:1000, ab8245, Abcam) diluted with the blocking solution at 4°C overnight. After rinsing with TBST for 3×10 minutes, the blots were incubated with HRP-conjugated secondary antibody in the blocking solution for 1 h at room temperature. After rinsing with TBST, the bands were visualized using an enhanced ECL detection kit (Santa Cruz Biotechnology). Relative intensity of each band compared with the GAPDH control was measured by Image J software (National Institute of Health).

qRT-PCR

OA side (left) of the L4-S5 segments spinal cord was dissected out as described for western blotting experiments. Total RNA was extracted from frozen tissues by the Trizol reagent (Invitrogen, CA, USA) in accordance with manufacturer's guidance. The reverse transcription of cDNA was carried out from total RNA samples with random primers using the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific, USA). Then, qRT-PCR amplification of each sample in triplicate was performed using SYBR® Green Real time PCR Master Mix (Toyobo Co. Ltd., Osaka, Japan) using the following conditions: initial denaturation at 94°C for 10 min, and 30 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 2 min, and followed by a final extension step at 72°C for 10 min. β-actin was selected as a reference for quantification, and the relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows:

IP-10, F 5'-GGGCCATAGGAAAACCTGAAATC-3' and R 5'-CATTGTGGCAATGATCTCA ACAT-3'; CXCR3, F 5'-TCGGCTCTGGTCTCTGCAA-3' and R 5'-GCTTATACAGGCCA GC AGGAA-3'; END, F 5'-CCCTATGGCCCTCCGCTGCCTCCTC-3' and R 5'-TTTTTTTTT TAAGTCTGAAAGGTTTTAT-3'; ENK, F 5'-CCTCCGACCTGCTGAAAGAG-3' and R 5'-TGC TTTCTGTTGGTGGCTAT-3'; DYN, F 5'-CATCAGC-CCAACCGCTCTAT-3' and R 5'-GGTA CAAAGACA-AGTTTCTCTGGATT-3'; β-actin forward: 5'-CAC-CATGTACCCTGGCATTG-3', R: 5'-TAACGCAACTA-AGTCATAGT-3' (Shanghai Institute of Biochemistry, China).

Statistical analysis

All experiments were repeated at least 3 times, with 3 replicates in each group. The data were analyzed using SPSS13.0 software and presented as means \pm standard error of mean (SEM). Statistical significance between different groups was compared with one-way ANOVA and $P < 0.05$ or $P < 0.01$ was considered as statistically significant.

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Disclosure of conflict of interest

None.

Address correspondence to: Yaochi Wu, Department of Acupuncture, Tuina and Traumatology, The Sixth People's Hospital Affiliated to Shanghai Jiaotong University, 600 Yishan Road, Shanghai 200233, China. Tel: +86-21-24058567; Fax: +86-21-24058567; E-mail: yochiwu@126.com

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