Original Article

Microglia are Involved in Pain Related Behaviors during the Acute and Chronic Phases of Arthritis Inflammation

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Abstract

Background: Pain is one of the main protests of inflammatory diseases, hence, understanding the mechanisms which involved in the induction and persistence of pain is essential. Microglia is a contributing factor in the onset and maintenance of inflammation. Increased microglial activation increases the level of central pro-inflammatory cytokines and the development of central sensitization following inflammation. The aim of this study was evaluated the relation of spinal microglia activity with pain related behaviors during Complete Freund's adjuvant (CFA)-induced inflammation.

Materials and Methods: Inflammation caused by subcutaneous injection of Complete Freund's adjuvant (CFA) in a single dose to the animal's right hind paw. The edema and hyperalgesia caused by inflammation, respectively, were measured by Plethysmometer and Radiant Heat, on days 0, 7, 14, and 21. Spinal Iba-1 protein expression was detected by Western blotting. Minocycline hydrochloride (Sigma, U.S.A) was administered intraperitoneal at a dose of 40 mg/kg daily.

Results: Our study findings indicated that CFA injection to the right hind paw of rats increased paw volume and hyperalgesia significantly during different stages of study, while Minocycline treatment significantly reduced paw volume and hyperalgesia. CFA injection into the right hind paw of the rat increases the expression of molecules ionized calcium binding adaptor molecule-1 (Iba-1) on different days of study, while Minocycline administration reduced spinal Iba-1 expression significantly compared to the CFA group.

Conclusion: The results of this study indicated significant roles of microglia activation in deterioration of pain related behaviors during different stages of CFA-induced inflammation. The steady injection of Minocycline (as a microglia inhibitor) could reduce the inflammatory symptoms.

Keywords: Inflammation, pain, microglia, minocycline

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder causing inflammation of the joints and surrounding tissues (1). Inflammation is

part of the non-specific immune response that occurs in reaction to any type of bodily injury. In some disorders, the inflammatory process -which under normal conditions is self-limiting- becomes continuous and chronic inflammatory diseases might develop subsequently (2).

Inflammatory responses in the peripheral and central nervous systems play key roles in the development and persistence of many pathological pain states (3). Much research in recent years focused on the role of microglia in maintenance of pain (4). Microglial cells represent the immune system at the spinal cord level and comprise as little as 5–20% of all glial cells in physiological functions (5, 6). Studies indicated followed the peripheral nerve injury, microglial cells are strongly activated in the animal neuropathic pain model both in the DRGs and at the spinal level (7). Microglia cells are sensitive to changes in their environment and quickly activated in response to pathogens and inflammation (8). Peripheral inflammation often results in inflammatory pain associated with hyperalgesia and allodynia (9).

Various inflammatory mediators in response to inflammation will be released from blood cells and damaged tissue including histamine, bradykinin and pro-inflammatory cytokines (10). Primary afferent nociceptor sensitization with cytokines is the consequence of damage to tissues (11). Increasing of excitatory signal transition from primary afferent fibers to dorsal horn neuron can cause central sensitization and pursuantly induction of hyperalgesia and allodynia (12). It was known that microglial cells are one of the important factors in the induction and maintenance of inflammation (13).

Acute inflammation in rats, after increases of inflammatory cytokines which induced by adjuvant can activates microglia in the lumbar spinal cord (14). Studies have shown microglia activation in response to inflammation and subsequent increase of pro-inflammatory cytokines, plays a key role in the development of central sensitization following peripheral nerve injury or inflammation (15, 16). Central pro-inflammatory cytokines primarily produced by microglia and have key role in central sensitization and chronic pain (17, 18). Central cytokines through presynaptic and postsynaptic mechanism increases the activity of excitatory synapses in the spinal cord, resulting in the development of central sensitization (12, 19).

Inflammation caused by plantar injection of complete Freund's adjuvant (CFA), commonly used as an animal model for acute and chronic inflammatory pain (20). Our previous studies have shown that CFA induced inflammation reduces peripheral afferents stimulation threshold in the spinal cord, which ultimately leads to hyperalgesia and edema during first week after the intervention. CFA induced inflammation, is a two-phase model. The first phase (phase of inflammation) is associated with increased pain due to the presence of pro-inflammatory cytokines, while in the second phase (Phase of arthritis) dramatically reduced hyperalgesia (21). Minocycline, a tetracycline-like anti-biotic, has been used as a microglial activation inhibitor and shown to ameliorate several neurodegenerative conditions (22). Then, based on the potency of minocycline in inhibition the microglia activity, in this study, the effects of minocycline on behavioral aspects of inflammatory arthritic pain induced by CFA was investigated.

Methods

Animals

Adult male Wistar rats with an average weight of 200-220 gr were used for this study. Rats were housed at a temperature of 22±2°C with a 12h light–dark cycle, and fed food and water ad libitum. The animals were allowed to habituate to the housing facilities for at least 1 week before the experiments were begun. All procedures were approved by the local ethics committee for the use of animals in research, and the guidelines of the International Association for the Study of Pain (23).

Local paw inflammation induction

Complete Freund's adjuvant (CFA), heat-killed mycobacterium tuberculosis (Sigma, St. Louis, MO, USA) suspended in a sterile mineral oil (10 mg/ml), was injected on Day 0 subcutaneously into the plantar surface of the right hind paw. Right hind paw of the control rats was only injected with sterile mineral oil (100μl). First day after CFA injection unilateral inflammation was established in injected hind paw, then first week was considered as inflammatory phase. The third week after intervention was arthritic

phase.

Assessment of CFA-induced inflammation and paw edema

Arthritis due to CFA injection was assessed by measurements of paw volumes pre- and post-injection (on days 0, 7, 14 and 21). Measurements (paw volume) were conducted by displacement of an electrolyte solution in a plethysmometer (model 7141; Ugo Basile; Comerio VA, Italy). The rats' hind paw were submerged up to the tibiotarsal joint in the electrolyte-filled Perspex cell of the plethysmometer. The volume of liquid displacement, which is correlated to the paw volume, was indicated on a digital displayer. Volume measurements were conducted twice for each paw, and the average volume displacement was calculated. The volumes measured on days post-injection were calculated as the percentage of the day 0 volume (24).

Thermal hyperalgesia assessment

Paw withdrawal latency (PWL) from noxious heat by using the plantar test (Ugo Basile, Verse, Italy) was assessed in different experimental and control rats on days 0 (before injection of CFA), 7, 14, and 21. Rats were placed in Plexiglas boxes for 10–15 min before testing in order to habituate to test environment. The heat source was positioned under the plantar surface of the affected hind paw and activated. PWL automatically recorded by digital timer which connected to the heat source. If the rat did not withdraw its paw from stimulus by 20s, the test was terminated and the rat was assigned this cut-off value. Withdrawal latency was measured three times for each hind paw at an interval of 5– 10 min and the mean latency was calculated. Then, the mean value for the affected paw (CFA-injected paw) was subtracted from that for the other paw and the result considered as the hyperalgesia sign in the injured paw.

Drug preparation

Minocycline hydrochloride (Sigma, U.S.A) was dissolved in 0.9% saline. It was administered intraperitoneal at a dose of 40 mg/kg daily (24).

Western blot analysis

After behavioral tests, western blot was used to measure the levels of Iba-1 (as a specific marker of microglial activation) of the sample isolated from lumbar spinal cord of rats. Briefly, the rats were killed rapidly in a CO_2 chamber. Then the lumbar spinal cord was quickly removed on ice, and homogenized (Brinkman polytron homogenizer, 20000 rpm for 30 s) in the lysis buffer containing proteinase inhibitors (Sigma). The extracted proteins were harvested for analysis and concentration of proteins are identified. Samples were separated by electrophoresis and the proteins transferred to poly vinylidene difluoride filters (PVDF) (Millipore). The status of non-specific bands on PVDF paper with incubation (90 minutes at 24°C) in blocking buffer blocked. Incubation was done with primary antibody in blocking buffer (Goat polyclonal to Iba1), (1:2000; Abcam/ ab5076) for 1 h at 24°C continues. Then membranes washed three times with TBST buffer (Tris-buffered saline with Tween 20) and then incubated with secondary antibody in blocking buffer (Anti-Goat (1:10,000); Abcam/CA) for 1 h at 24°C. The papers washed three times with TBST. The membranes were then incubated in stripping buffer and reported with beta-actin primary antibody (1:5000; Cell Signaling) as a loading control. The protein immune response can be seen on paper with identification system chemoimmunoluminens. To ensure the detection of microglia, the Iba-1 is measured relative to beta-actin. Bond concentration of densitometry using NIH Image with measured and reported. Each test is repeated 3 times for each group (25).

Experimental procedures

In order to determine the effect of minocycline on inflammatory pain model and the effectiveness of the treatment, a series of experiments were performed. Rats were randomly divided into different experimental groups, as follows: (a) CFA group, (b) CFA control group, (c) CFA + Minocycline (d) CFA+0.9% saline.

According to the study procedure, each group was divided into four subgroups based on different time points of the study (days 0, 7, 14, and 21) and there were 6 rats in each subgroup. Inflammation induction is done on day zero. The CFA control group was received sterile mineral oil once only (100μL) (S.C.). From the first day after CFA injection, experimental groups received the minocycline intraperitoneal (i.p.) 40 mg/kg daily. Normal saline as diluents of minocycline was used in control groups (showed as a 0.9% saline + CFA group) and the

assessments were fully performed as the minocycline + CFA groups. At the end of each period (days 0,7, 14, 21), after conducting behavioral tests, the rats were killed rapidly, then, lumbar spinal cord of rats was isolated on ice for western blot, used to measure the levels of Iba-1 (as a specific marker of microglial activation).

Statistical analysis

Results are presented as the Mean ± SEM. Data was analyzed by one way analysis of variance (ANOVA), followed by Post hoc Tukey's test for multiple comparison. Unpaired Student's *t*-test was used to determine significant differences between the groups. An effect was determined to be significant if the p-value was less than 0.05. The p value of less than 0.05 was considered as statistically significant.

Results

Variation of Paw volume during different stages of study

CFA injection into the right hind paw caused a significant increase in paw volume (edema) which was continued until the 21st day of the study. Right injected paw volume showed a significant increase on days 7, 14, and 21 after CFA injection compared to day 0 and with CFA control group (p≤0.01 for day 7 and p≤0.001 for day 14 and 21). There were no significant differences in reduction in paw volume following injection of sterile mineral oil to the rat's right hind paw on different days of the study relative to baseline in the CFA control group (Fig. 1).

Daily administration (i.p.) of Minocycline in the CFA+ Minocycline group significantly reduced paw volume. Minocycline injection in the group significantly reduced paw volume compared to the same days in CFA group throughout this study (p≤0.001 for days 14 and 21). In comparison between the groups CFA+ Minocycline and CFA, the results illustrated that reduction of paw edema following the injection of Minocycline, at day 21 was higher than day 14 substantially (p≤0.01). However Minocycline treatment decremented paw volume in the CFA+ Minocycline group, so that at day 21 of the experiment, no significant distinction in paw volume in CFA+ Minocycline group compared with CFA control group was perceived (Fig. 2). In addition,
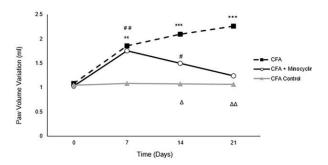

Fig. 1. CFA injection caused significant edema in affected paw which continued till 21 days of study. Long-term Minocycline treatment significantly reduced inflamed paw volume. Results presented as Mean ± SEM (n=6/group).
** p≤0.01 and *** p≤0.001 for comparing the paw volume variations between baseline and different days of the study in CFA group. # p≤0.05 and # # p≤0.01 for comparing the paw volume variations between day 0 and different days of the study in CFA+ Minocycline group. Δ p≤0.05 and ΔΔ p≤0.01 for illustrating the variations in paw volume at day 14 and day 21 compared to day7 in the CFA+ Minocycline group.
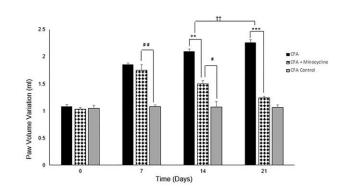

Fig. 2. CFA injection increased paw volume significantly, while Minocycline administration caused a remarkable reduction in paw volume compared with CFA group. Results stated as Mean± SEM (n=6/group). ** p≤0.01 and *** p≤0.001 for comparing the paw volume variations between CFA and CFA+ Minocycline groups in identical days. # p≤0.05 and # # p≤0.01 for comparing the paw volume variations between CFA+ Minocycline and CFA control groups in identical days. †† p≤0.01 for comparing the differences in paw volume alterations in CFA and CFA+ Minocycline groups at day 14 compared to day 21.
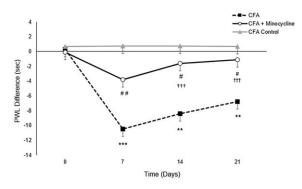
Fig. 3. CFA injection into the hind paw of rats cause significant hyperalgesia at 7th day but, hyperalgesia reduced at 14th and 21st days, and long-term injection of Minocycline considerably decremented thermal hyperalgesia. Results presented as Mean ± SEM (n= 6/group).
** $p \leq 0.01$ and *** $p \leq 0.001$ for comparing the PWL variations in CFA group between day 0 and different time points of the study. # $p \leq 0.05$ and # # $p \leq 0.01$ for comparing the PWL variations in CFA+ Minocycline group between day 0 and different time points of the study. ††† $p \leq 0.001$ for comparing the changes in PWL in the CFA+ Minocycline group at day 14 and day 21 compared to day 7.

there were no significant differences in paw volume of rats during 21 days of the study in CFA+0.9% saline (as a vehicle of Minocycline) group (Hence, the results of the CFA+0.9% saline groups are not shown graphically).

Thermal hyperalgesia Variations during different stages of study

Thermal hyperalgesia varied in the right (injected) paw during different stages of inflammation. Hyperalgesia significantly increased on day 7 after CFA injection compared to day 0 in the CFA group ($p<0.001$), but our results also indicated that inflammation continuation caused hyperalgesia to decrease on days 14 and 21. However, there was still a significant increase compared to day 0 ($p \leq 0.01$). There were no significant differences in reduction of thermal hyperalgesia following injection of sterile mineral oil to the rat' right hind paw on different days of the study relative to baseline in the CFA control group. Daily administration (i.p.) of Minocycline in the CFA+ Minocycline group reduced hyperalgesia (Fig. 3).

Minocycline injection in the CFA+

Fig. 4. CFA injection considerably raise thermal hyperalgesia while Minocycline administration reduced thermal hyperalgesia compared to the CFA group. Results stated as Mean± SEM (n=6/group).
** $p \leq 0.01$ and *** $p \leq 0.001$ for Comparing the PWL variations between CFA and CFA+ Minocycline groups in identical days. # # # $P \leq 0.001$ for comparing the differences in PWL variations in CFA and CFA+ Minocycline groups at days 14 and 21 compared to day 7.

Minocycline group significantly reduced thermal hyperalgesia compared to the same days in CFA group throughout this study ($p \leq 0.01$ for day 7 and $p \leq 0.001$ for days 14 and 21). In comparison between the CFA and CFA+ Minocycline groups, the results illustrated that the rate of reduction of thermal hyperalgesia following the injection of Minocycline, at days 21th and 14th after CFA injection between these two groups was higher than day 7 ($p \leq 0.001$). Continuing injection of Minocycline decremented PWL in CFA+ Minocycline group, so that at day 21 of the study, no significant difference in PWL in CFA+ Minocycline group compared with CFA control group was observed (Fig. 4). Additionally, there were no significant differences in PWL of rats during 21-days of the study in CFA+0.9% saline (as a vehicle of Minocycline) group (Hence, the results of the CFA+0.9% saline groups are not shown graphically).

Variation in spinal Iba-1 expression during different stages of study

In order to check the Iba-1 protein expression in the obtained tissues from the lumbar spinal cord of all experimental groups, we used polyclonal antibody to detect Iba-1 protein as a marker of microglia activity in the spinal cord. The analysis showed similar bands with molecular weight of almost 17

KDa which was the same as the manufacturer's instructions. Immunospecificity was confirmed by the absence of immunoreactive bands when the membrane was preincubated with an antigenic peptide prior to antibody incubation (data not shown) (26). Following quantification of mOR-immunoreactive bands, membranes were stripped and re-probed for β-actin as a loading control (43 kDa). To normalize differences in protein-loading, all data were expressed as Iba-1/β-actin ratio.

Our results confirm that, on the lumbar part of the spinal cords obtained from the CFA-induced inflammation rats, spinal Iba-1 protein expression observably increased on days 7, 14, and 21 compared to day 0 of the study ($p \leq 0.001$, $p \leq 0.001$ and $p \leq 0.01$ respectively) (Fig. 5). There was no significant difference in Iba1 expression in the CFA control group. In the CFA+ Minocycline group, CFA injection into the right hind paw caused a significant increase in Iba-1 expression on day 7 compared to
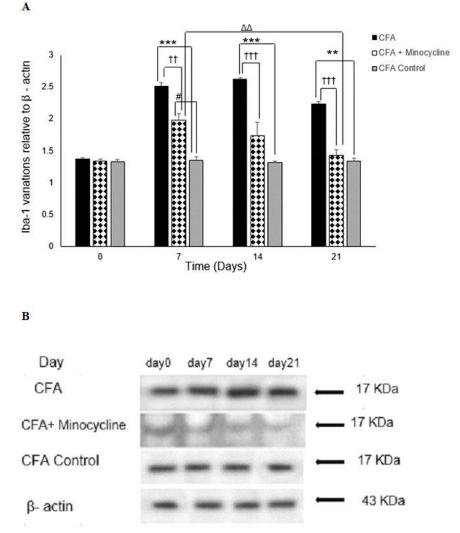

Fig. 5. CFA injection into the hind paw of rats cause significant hyperalgesia at 7th day but, hyperalgesia reduced at 14th and 21st days, and long-term injection of Minocycline considerably decremented thermal hyperalgesia. Results presented as Mean ± SEM (n= 6/group).
** $p \leq 0.01$ and *** $p \leq 0.001$ for comparing the PWL variations in CFA group between day 0 and different time points of the study. # $p \leq 0.05$ and # # $p \leq 0.$ 01 for comparing the PWL variations in CFA+ Minocycline group between day 0 and different time points of the study. ††† $p \leq 0.001$ for comparing the changes in PWL in the CFA+ Minocycline group at day 14 and day 21 compared to day 7.

day 0 (p<0.05). There was no significant difference in Iba-1 expression between days 14 and 21 compared to day 0 (data not shown). In addition, there were no significant differences in Iba-1 expression in rats during 21-days of the study in CFA+0.9% saline (as a vehicle of Minocycline) group (Hence, the results of the CFA+0.9% saline groups are not shown graphically). There was a significant difference in Iba-1 expression on days 7, 14, and 21 between CFA+ Minocycline and CFA groups (p<0.001, p<0.001, and p<0.01, respectively) (Fig.5).

Discussion

In this study, we show that there is relationship between spinal microglia activity and pain behavioral symptoms during adjuvant-induced inflammation. In this study plantar injection of CFA induced paw inflammation and edema which continued up to day 21 after the CFA injection. Hyperalgesia elevated considerably on day 7 after the CFA injection, but the continuity of inflammation declined hyperalgesia notably on days 14 and 21 of the study compared to day 7.

Arthritis model induced by CFA in rats is an inflammatory model extensively used in etiopathogenic investigational drug and molecular studies due to its similarity to human RA and assay the pathophysiological and pharmacological changes during human RA (27). Intraplantar injection of inflammatory agents such as CFA causes elevated firing of peripheral afferents in the spinal cord, leading to hyperalgesia (28). Sensitization of the primary afferent nociceptive fiber during inflammation is an important factor in the creation and development of hyperalgesia (11). Pursuant to Hargreaves study, rats due to its inflammation induced by plantar injection of CFA, had withdrawn his affected paw from the thermal stimuli, but some other studies have shown that motor behaviors subsequent of the inflammation are normal and no notable changes in motor activities are observed (29). A study illustrated that plantar injection of CFA incremented hyperalgesia and edema from 24 hours after the CFA injection and continued up to the first week after the injection (30). Thermal hyperalgesia never happened in the contralateral paw during arthritis induced by CFA adjuvant. Injection of CFA

into the one hind paw may have a pivotal role in the induction of hyperalgesia which occurs only in the ipsilateral paw, however the reasons for the absence of hyperalgesia in the contralateral paw should be clarified (31).

The results of this study indicated the role of Minocycline in reducing edema and hyperalgesia during different stages of inflammation caused by CFA. The continuing injection of Minocycline could reduce the inflammatory symptoms. Because these results suggest an association between microglial activation, which is known to contribute to pain after peripheral nerve injury, and hyperresponsiveness of pain related behaviors to peripheral stimulation and chronic pain after CFA injection. We downregulated pharmacologically activation of microglia with the inhibitor minocycline. After delivery of minocycline, we showed reductions in behavioral concomitants of pain, suggesting a role of microglia in the active modulation of ongoing below-level pain after CFA injection. The precise role of activated microglia in CFA induced inflammatory pain has not been studied previously, and our finding of a contribution of activated microglia to maintaining chronic pain after CFA injection is novel. There is a body of literature that suggests that microglia are involved in the initial phase of development of chronic pain after peripheral injury, but the role of microglia in its ongoing maintenance of pain has not been reported. Microglial activation occurs in response to CNS trauma, ischemia, tumors, neurodegeneration and immunogenic components of viruses and bacteria (32). Glial activation may enhance neuronal transmission of nociceptive information. One possible mechanism for activated glia to induce hyperalgesia and allodynia is through the release of pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6 (33). In line with these findings, we also observed the increased thermal hyperalgesia and edema with glial activation following the peripheral administration of CFA.

Conclusion

This study showed that peripheral inflammation induced by CFA causes glial activation and deterioration pain related behaviors. The steady injection of Minocycline (as a microglia inhibitor)

could reduce the inflammatory symptoms. However, further studies are needed to evaluate the relationship between microglia activity and central cytokines on different aspects of inflammation.

Acknowledgment

Conflicts of Interest

The authors declare that they have no conflict of interest.

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