Intrathecal minocycline suppresses itch-related behavior and improves dermatitis in a mouse model of atopic dermatitis

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Abbreviations: AD, atopic dermatitis; Dfb, Dermatophagoides farinae body; SD, standard deviation; TEWL, transepidermal water loss

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ABSTRACT

Chronic and intractable itch may occur due to abnormal neuronal firing in both the peripheral and central nervous systems. Recently, it has been demonstrated that spinal glial cells, such as microglia and astrocytes, are involved in modulation of neuropathic pain. However, the roles of spinal glial cells in intractable itch of skin diseases, such as atopic dermatitis (AD), are currently unknown. This study was performed to examine the antipruritic effects of lumbar intrathecal administration of minocycline, an inhibitor of microglial activation, in NC/Nga mice, AD-like model. Dermatitis was induced in mice by repeated application of *Dermatophagoides farinae* body (Dfb), and numbers of scratching bouts and spinal Iba1-immunoreactive (Iba1+) microglia were increased in the Dfb-NC/Nga mice compared with those in control mice. In pharmacological experiments, intrathecal minocycline dose-dependently reduced scratching bouts, concomitant with decreased number of spinal Iba1+ microglia in the Dfb-NC/Nga mice. Intrathecal minocycline also improved the dermatitis and skin barrier function in the Dfb-NC/Nga mice. Additionally, Oral administration of high-dose minocycline improved dermatitis and showed a tendency to reduce the numbers of scratching bouts and spinal Iba1+ microglia in the Dfb-NC/Nga mice. Therefore, spinal microglia may be one of the therapeutic targets for the intractable itch associated with AD.
TO THE EDITOR

Itch (or pruritus) is a common, sometimes unpleasant sensation that elicits an urge to scratch. Chronic pruritus (defined as itch lasting for > 6 weeks) found in patients with dermatological diseases including atopic dermatitis (AD) is often resistant to conventional treatments, including antihistamines. Such itch is a clinical problem that reduces the quality of life in patients (Warlich et al., 2015). However, the mechanisms underlying chronic and intractable itch have not been clarified.

There is recent accumulating evidence from pain research that spinal microglial activity plays a role in the pathogenesis of neuropathic pain. Neuropathic pain in animal models is ameliorated by intrathecal administration of minocycline, an inhibitor of activated microglia (Tikka and Koistinaho, 2001; McMahon et al., 2005). Meanwhile, the role of spinal microglia in the development of chronic and intractable pruritus, such as AD, has not been fully elucidated. Additionally, the effect of minocycline on pruritus of AD is currently unclear. We therefore investigated the antipruritic effect of minocycline on AD using NC/Nga mice, a model of AD (Tanaka et al., 2012).

Ten-week old male NC/Nga mice (Charles River Laboratories Japan, Yokohama, Japan), were maintained under clean conditions. All animal procedures were approved by the Animal Care and Use Committee of Juntendo University Graduate School of Medicine. We used a Dermatophagoides farinae body (Dfb) ointment-induced AD-like mouse model (Dfb-NC/Nga) as described (Yamamoto et al., 2009). Dermatitis was induced by repeated application of Dfb ointment (Biostir, Kobe, Japan) twice a week for 3 weeks (Figure 1a). The severity of skin lesions was graded according to criteria described (Matsuda et al., 1997). After induction, scratching behavior was monitored for 12 hours using a SCLABA®-Real system (Noveltac, Kobe, Japan) as described (Tanaka et al., 2012). Under sevoflurane anesthesia, transepidermal water loss (TEWL) was measured using a Tewameter® TM210 (Courage & Khazaka Electronic, Cologne,
Germany). Dfb-NC/Nga mice showed significant increases in number of scratching bouts, dermatitis score and TEWL value (Figure S1a-c). Distribution of spinal microglia in the Dfb-NC/Nga mice was examined immunohistochemically with an antibody against Iba1, a microglial maker (Ito et al., 1998). Increased numbers of spinal Iba1-immunoreactive (Iba1+) microglia were found in Dfb-NC/Nga mice compared with control mice (Figure S1d-f).

We next examined the effects of minocycline on scratching behavior and dermatitis in Dfb-NC/Nga mice. Minocycline (Sigma-Aldrich, St.Louis, MO) was dissolved in sterile saline, and filtrated using sterile syringe filters. Intrathecal administration of minocycline was performed according to the previously described method (Hylden et al., 1980). Mice with a dermatitis score > 5 were treated by intrathecal administration of sterile saline (vehicle) or minocycline at doses of 5 or 50 µg in 5 µL saline three times a week for 2 weeks (Figure 1a). In the treatment phase, scratching behavior was monitored for 12 hours once a week (Figure 1a). The number of scratching bouts was significantly decreased after treatment for 1 week in 50 µg minocycline-treated mice compared with control mice (Figure 1b). Minocycline was more effective in alleviating scratching behavior after treatment for 2 weeks (Figure 1b). We also monitored locomotion activity in the mice for 12 hours using SCLABA®-Real software (Noveltec) and found no significant difference among the three groups (Figure 1c). The numbers of Iba1+ microglia in the posterior area of the spinal cord were also significantly decreased after treatment for 2 weeks in 50 µg minocycline-treated mice compared with control mice (Figure 1d and 1e).

Dermatitis score was further assessed after each Dfb application in the treatment phase (Figure 1a). In 50 µg minocycline-treated mice, dermatitis score was significantly decreased at 1 week after treatment, with improvement sustained throughout the experiment (Figure 2a and 2b). The TEWL value in dorsal skin lesions in the 50 µg
minocycline-treated mice was also decreased after 2 weeks of treatment (Figure 2c).

We also examined the effects of oral minocycline on the AD-like symptoms shown in Figure S2a. Oral high-dose minocycline improved dermatitis in the Dfb-NC/Nga mice (Figure S2b). The systemic administration showed a tendency to reduce the numbers of scratching bouts (Figure S2c) and spinal Iba1+ microglia (Figure S2d).

The present study showed that intrathecal minocycline dose- and time-dependently suppressed scratching behavior in Dfb-NC/Nga mice, concomitant with decreased numbers of Iba1+ microglia. Oral minocycline also had a tendency to suppress scratching behavior and decrease numbers of Iba1+ microglial (Figure S2). Clinically, minocycline is used as a broad-spectrum tetracycline antibiotic (Garrido-Mesa et al., 2013). In addition to anti-inflammatory and immunomodulatory effects of minocycline (Garrido-Mesa et al., 2013), it inhibits p38 mitogen-activated protein kinase in the microglia and thereby prevents their proliferation (Tikka and Koistinaho, 2001). Minocycline was also shown to reverse microglial reactivity in neuropathic pain models (LeBlanc et al., 2011). A more recent study suggested that spinal reactive astrocytes play a role in itch sensitization of AD model mice (Shiratori-Hayashi et al., 2015). Therefore, although we cannot exclude effects of minocycline on the astrogliosis, the anti-scratching effect of minocycline may be at least partly associated with inhibition of microglial reactivity. This may be supported by a recent study indicating that scratching activates spinal microglia in acute pruritic model mice (Zhang et al., 2015a).

Moreover, removing the claws of NC/Nga mice, thus preventing scratching, successfully inhibited the induction and progression of dermatitis (Hashimoto et al., 2004). We found that intrathecal minocycline improved skin barrier function (Figure 2c) and dermatitis in Dfb-NC/Nga mice (Figure 2b). Dermatitis was also improved by oral high-dose minocycline (Figure S2b). Thus, in addition to the anti-inflammatory effect, an anti-scratching effect by intrathecal minocycline administration may contribute to
improvement of dermatitis through prevention of skin barrier damage.

A recent study also indicated that intrathecal administration of minocycline suppressed scratching behavior in a contact dermatitis (CD) mouse model induced by repeated applications of 2,4-dinitrofluorobenzene (DNFB), although the anti-scratching effect was not sustained after treatment (Zhang et al., 2015b). Thus, given that the pathophysiology and pathological process of AD are different from those of CD induced by haptens, such as DNFB (Kabashima, 2013), the pharmacological efficacy may reflect differences in the degree of microglial activation.

**Conflict of interest**

The authors declare they have no conflicts of interest.

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**Supplementary material**

**Supplemental data. Additional materials and methods.**

**Supplementary Figure S1. Distribution of Iba1+ microglia in the spinal cord of Dfb-NC/Nga mice.**

**Supplementary Figure S2. Effects of oral minocycline on dermatitis, scratching behavior and spinal Iba1+ microglia in Dfb-NC/Nga mice.**
References


Figure 1. Torigoe et al.

(a) Induction phase  Treatment phase

Dfb

Intrathecal injection (i.t.)

Behavior observation

(b) Number of scratching bouts

Saline (control)  5 μg minocycline  50 μg minocycline

(c) Locomotion activity [cm]

Before 1 2

Weeeks after treatment

(d) Saline (control)  Minocycline 50 μg i.t.

(e) Number of flii's minocytes in paraffin section of sprue site

saline (control)  5 μg  50 μg
Figure 2. Torigoe et al.

(a) 

Before treatment 

Saline (control) 5µg minocycline (i.t.) 50µg minocycline (i.t.)

After treatment 

(b) 

Dermatitis score 

Time after treatment 

Before 1 2 3 4 5 6

(c) 

TEMW [g·m⁻²·h⁻¹] 

(at 2 weeks after treatment) 

Saline (control) 5 µg 50 µg i.t. 

*
**Figure legends**

**Figure 1. Effects of intrathecal minocycline on scratching behavior in Dfb-NC/Nga mice.** (a) Experiment schedule. After induction of dermatitis, saline or minocycline was administered intrathecally three times per week for 2 weeks. (b) Scratching bouts were significantly reduced after treatment with 50 µg minocycline for 1 and 2 weeks. (c) No significant difference in locomotion activity was observed among the groups. (d) Images of spinal Iba1-immunoreactive (Iba1+) microglia (green) in saline- and 50 µg minocycline-treated mice. Scale bar; 100 µm. (e) The numbers of spinal Iba1+ microglial significantly decreased after treatment with 50 µg minocycline for 2 weeks. Data (mean ± SD, n = 7-8 per group) were compared by one-way analysis of variance and Bonferroni’s method. ***P < 0.001, ****P < 0.0001 vs. control.

**Figure 2. Effects of minocycline on dermatitis and transepidermal water loss (TEWL) in Dfb-NC/Nga mice.** (a) Representative images of lesional skin in Dfb-NC/Nga mice before and after two weeks of treatment with minocycline. (b) In the 50 µg minocycline-treated mice, dermatitis score was significantly decreased after 1 week of treatment, with improvement sustained for 2 weeks (P < 0.01). (c) The TEWL value in the dorsal skin lesions significantly improved after 2 weeks of treatment in 50 µg minocycline-treated mice. Data (mean ±SD, n = 7-8 per group) were compared by one-way analysis of variance and Bonferroni’s method. *P < 0.05, **P < 0.01 vs. control.
Supplementary data

Supplemental materials and methods

Animals
Male NC/Nga mice (10-week old; Charles River Japan, Yokohama, Japan) were maintained in the experimental animal facility of Juntendo University Graduate School of Medicine under controlled temperature (22 - 24°C), humidity (50 ± 5%) and light (light on 8:00 - 20:00). Food and tap water were provided *ad libitum*. All animal procedures were approved by Animal Care and Use Committee of Juntendo University Graduate School of Medicine.

Induction of atopic dermatitis-like symptoms
Dermatitis was induced by application of Dfb ointment (Biostir, Kobe, Japan) twice per week for three weeks. Severity of skin lesion was graded according to criteria described (Matsuda et al., 1997).

Intrathecal treatment with minocycline
After three weeks of Dfb application, mice with a dermatitis score > 5 were selected and divided into three groups: group 1, saline (*n* = 7); group 2, 5 µg minocycline (*n* = 8); group 3, 50 µg minocycline (*n* = 8). Minocycline (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline, and filtrated through a sterile syringe filter. Intrathecal administration was performed as described (Hylden JL et al., 1980). Briefly, 5 µL solution was intrathecally injected into the lumbar spinal area (L5/L6) using a 25 µL Hamilton syringe with 33-gauge needle under sevoflurane anesthesia. Slight flinching of the tail was considered indicative of successful intrathecal administration. The procedure was conducted three times per week for 2 weeks.
**Oral treatment with minocycline**

After three weeks of Dfb application, mice with a dermatitis score > 5 were selected and divided into three groups: group 1, saline \((n=6)\); group 2, 5 mg kg\(^{-1}\) minocycline \((n=6)\); group 3, 25 mg kg\(^{-1}\) minocycline \((n=6)\). Saline or minocycline (5 or 25 mg kg\(^{-1}\) in 200 \(\mu\)L saline) was administered orally (p.o.) by syringe twice per day for 3 weeks. Minocycline was prepared as described above.

**Analysis of scratching behavior**

Scratching behavior was recorded and analyzed using a SCLABA\(^{\text{®}}\)-Real system (Noveltec Inc., Kobe, Japan). In brief, mice were put into an acryl cage and after acclimatization period of at least an hour scratching behaviour was recorded continuously for 12 hours once a week, with no human present.

**Clinical skin severity score**

Severity of skin lesion was graded according to criteria described (Matsuda et al., 1997). Severity scoring was performed before applying Dfb ointment twice per week in induction and treatment phases. Clinical features of AD-like lesions (erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness) were graded as 0 (none), 1 (mild), 2 (moderate) and 3 (severe), with a maximum score being 12 in total.

**Measurement of transdermal water loss (TEWL)**

TEWL was measured under sevoflurane anesthesia on the dorsal skin lesion three times per week using a tewameter\(^{\text{®}}\) TM210 (Courage & Khazaka Electronic, Cologne, Germany) and the median value of three measurements at each time point documented.
**Immunohistochemistry**

Mice were deeply anesthetized with intraperitoneal administration of somnopentyl and perfused through the left ventricle with phosphate saline buffer (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, the cervical and upper thoracic spinal cord was harvested, post-fixed in 4% paraformaldehyde solution for 4 hours, and kept in PBS containing 20% sucrose overnight at 4°C. Samples were cut transversely into 20 µm-thick frozen sections on a cryostat, CM3050S® (Leica Biosystems Nussloch, Wetzlar, Germany). Six sections, as one set per animal, were collected for semi-quantitative measurement and mounted onto silane-coated glass slides. After blocking in PBS containing 5% normal donkey serum, 2% bovine serum albumin and 0.2% Triton X-100 (blocking solution), was used as a primary antibody. The sections were incubated with rabbit polyclonal anti-Iba1 (1:500, Wako Pure Chemical, Osaka, Japan) overnight at 4°C. Then, the sections were washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated with Alexa Fluor 488-conjugated species-specific secondary antibody (1:300; Invitrogen, Danvers, MA) for one hour at room temperature. After washing with PBS-T, the sections were mounted in Vectashield® mounting medium (Vector Laboratories, Burlingame, CA).

For semi-quantitative measurement, the number of Iba1⁺ microglia was counted in the posterior area of the spinal cord, behind the central canal, in each mouse.

**Statistics**

Data are expressed as mean ± standard deviation (SD). The statistical significance of differences among data of control and treated mice was determined by one-way analysis of variance, and Bonferroni’s method or unpaired *t* test using GraphPad Prism 6 (GraphPad Software, San Diego, CA) with *P* < 0.05 taken to indicate statistical significance.
Supplemental figures

Supplemental Figure S1. Distribution of Iba1+ microglia in Dfb-NC/Nga mice spinal cord. (a) The number of scratching bouts in 12 hours was significantly increased after repeated application of Dfb ointment. In Dfb-NC/Nga mice, (b) dermatitis score and (c) TEWL value significantly increased after repeated application of Dfb ointment including preconditioning by sodium dodecyl sulfate (SDS). (d) A representative image of spinal Iba1+ microglia (green) in SDS-NC/Nga mice (control) (e) A representative image of spinal Iba1+ microglia (green) in Dfb-NC/Nga mice. Scale bar, 100 µm. (f) The number of Iba1+ microglia in the posterior area of the spinal cord was significantly increased in Dfb-NC/Nga mice compared with that in SDS-NC/Nga mice. Data (mean ± SD, n = 6 animals) were compared by unpaired t test. **P < 0.01, ****P < 0.0001 vs. control.
Supplemental Figure S2. Effects of oral minocycline on dermatitis, scratching behavior and spinal Iba1+ microglia in Dfb-NC/Nga mice. (a) Experiment schedule. After the induction phase, saline or minocycline (5 or 25 mg per kg in 200 μL saline) was administered orally (p.o.) twice per day for 3 weeks. (b) In 25 mg kg$^{-1}$ minocycline-treated mice, the dermatitis score was significantly decreased after the 3-week treatment. (c, d) No significant difference was found among the three groups in numbers of scratching bouts or Iba1+ microglia in the posterior area of the spinal cord. Data (mean ±SD, n = 6 animals) were compared by one-way analysis of variance and Bonferroni’s method.