Etanercept attenuates pain-related behavior following compression of the dorsal root ganglion in the rat

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Abstract

Purpose TNF α is an inflammatory mediator related to neuropathic pain including sciatica. Much basic research suggests that anti-TNF α therapy may be useful for the treatment of sciatica. The purpose of this study was to clarify the effects of etanercept in a dorsal root ganglion (DRG) compression model.

Methods Adult male Sprague-Dawley rats (200–250 g, $n = 60$) were used. An L-shaped stainless rod was used to compress the left L5 DRG in the saline and etanercept groups. No rod was used in the sham group. In the etanercept group, 1 mg of etanercept was applied locally onto the DRG at the end of surgery. Saline was applied in the saline and sham groups. On day 3 and day 7 after surgery, the number of ED1-immunoreactive (IR) cells (macrophages) in the DRG was calculated by immunohistochemical methods ($n = 6$). In addition, double-immunofluorescence labeling for ED1 and TNF α was performed. Behavioral testing with von Frey filaments and a heat stimulator was performed ($n = 12$).

Results ED1-IR cells in the DRG significantly increased in the control group compared with the sham group ($p < 0.05$). Some ED1-IR cells were co-labeled for TNF α . In the etanercept group, decrease in mechanical threshold was significantly inhibited compared with the saline group ($p < 0.05$). Thermal hyperalgesia was observed in the control group, but in neither the sham nor etanercept group ($p < 0.05$).

Conclusion Etanercept attenuated the pain-related behavior induced by DRG compression. These findings suggest that mechanical effects on the DRG might be reduced by etanercept in addition to the effects on nucleus pulposus in lumbar disc herniation.

Keywords Sciatica · Dorsal root ganglion · Tumor necrosis factor- α · Etanercept · Macrophage

Introduction

Sciatica is a major symptom of degenerative lumbar spinal disorders such as disc herniation and spinal canal stenosis. Mechanical compression of the spinal nerve root and/or the dorsal root ganglion (DRG) is one important pathophysiological factor in such symptoms, while chemical irritation induced by nucleus pulposus or nerve injury may be another. Mechanical compression of the nerve root and/or DRG induces edema and thrombosis of nerve tissue [28], decrease in blood flow, and increase in endoneurial pressure [13], as well as pain-related behavior and hyperexcitability of DRG neurons [11, 18, 33, 39]. Compression of the DRG produces long periods of repetitive firing [10, 34]. We previously reported that compression of the DRG in rats produced pain-related behavior and structural changes in the spinal cord [38]. These findings suggest that mechanical compression of the DRG is closely related to pain. On the other hand, it has already been reported that nucleus pulposus could induce pain without any mechanical compression of the nerve and that TNF α plays a very important role in the mechanism of pain induced by nucleus pulposus [14, 19, 24–27, 29, 35]. Peripheral nerve injury induces TNF α and TNF α receptor expression in the Schwann cells in injured nerves, satellite cells, and neurons

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in the DRG, as well as astrocytes in the spinal cord [23, 37]. TNF α antagonists attenuate the pain-related behavior observed in experimental models of neuropathic pain including the DRG compression model [9, 30–32]. TNF α and inflammatory responses may thus play important roles in the neuropathic pain induced by nerve injury and DRG compression.

It has been reported that macrophage infiltration increased in the DRG after peripheral nerve injury and application of nucleus pulposus [7, 12, 17, 21], and that thermal hyperalgesia after chronic constriction nerve injury was reduced in mice, with inhibition of macrophage infiltration [16, 22]. These findings suggest that macrophages may play important roles in the neuropathic pain that occurs following nerve injury.

Etanercept is a soluble TNF α receptor-Fc protein and neutralizes TNF α [20]. Macrophages in the synovium of joints affected by rheumatoid arthritis decrease in number due to apoptosis following anti-TNF α therapy [2]. In addition, TNF α inhibitor reduces infiltration of macrophages with myeloid-related proteins 8 and 14 from the peripheral circulation [5]. A previous experimental study suggested that etanercept reduced neuropathic pain [32]. Etanercept may thus reduce macrophage infiltration into nerve tissue, and alleviate neuropathic pain.

The objective of this study was, first, to observe the changes in macrophages and TNF α in compressed DRG. Secondly, we investigated the effects of etanercept on pain-related behavior following DRG compression using behavioral testing.

Materials and methods

Seventy-two adult male Sprague-Dawley rats (body weight 220–300 g) were used in this study. All animal experiments conformed to the regulations of the Animal Research Committee of Fukushima Medical University, which

accorded with the Guidelines on Animal Experiments of Fukushima Medical University and Japanese Government Animal Protection and Management Law (No. 105).

Surgical procedure

Dorsal root ganglion compression was induced as described in our previous report [38]. Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). The left L5 lamina was exposed and a drill hole (2.0×1.5 mm) was made in the lamina using an electric drill. In the compression group ($n = 48$), an L-shaped stainless steel rod with a diameter of 0.5 mm and a side length of 2 mm was inserted into the drill hole distally along the DRG and nerve root. The DRG and proximal part of the nerve root were compressed with the side of the L-shaped rod (Fig. 1). In the sham group ($n = 24$), only the drill hole was made without insertion of the rod. The compression group was divided into two subgroups: a saline group (non-treatment group, $n = 24$) and an etanercept group (treatment group, $n = 24$). In the etanercept group, a piece of Spongel (Yamanouchi Pharmaceutical, Tokyo, Japan) containing 1 mg/20 μ l etanercept (Wyeth K.K, Tokyo, Japan) was applied to the left L5 DRG at the end of surgery. The amount of etanercept was decided according to a previous report [30]. In the saline group, a Spongel containing 20 μ l saline was applied instead of etanercept to evaluate the effects of etanercept on DRG compression. In the sham group, Spongel with saline was also applied. We set the sham group as no compression with no treatment for comparison with the saline group (DRG compression with no treatment) in order to determine the effects of DRG compression.

Immunohistochemistry for ED1

ED1 was selected to observe macrophages in the DRG according to previous reports [6, 12, 17, 21]. Our

Fig. 1 The schematic figure (a) and photo (b) show the surgical procedure for inducing compression of the left L5 DRG. An L-shaped stainless steel rod was inserted into the drill hole distally along the nerve root and DRG. *Plus symbol* drill hole with inserted rod, *asterisk* left L5 inferior articular process, *hash symbol* L5 spinous process

preliminary study showed ED1-IR cells were increased at day 3 and day 7 but not day 14 or 28. Therefore, we observed ED1-IR cells at day 3 and day 7. Thirty-six rats were used in this experiment (each group: $n = 12$). At 3 and 7 days after surgery, six rats each were deeply anaesthetized with inhalation of ether and subjected to intracardiac perfusion with 200 ml saline followed by 300 ml of paraformaldehyde in 0.1 M PBS. Left L5 DRGs were then removed and postfixed overnight in the same fixative. Tissue was embedded in paraffin and cut into transverse 6 μm sections. The sections were mounted on slides, deparaffinized with xylene, rehydrated in a graded ethanol series, and processed immunohistochemically. Tissue sections were preincubated in 0.3% H_2O_2 in PBS for 10 min and then washed in PBS. The tissue sections were incubated for 30 min at room temperature in a blocking solution of 1.5% normal horse serum in 0.1 M PBS and then incubated overnight at 4°C in mouse anti-monocyte/macrophage antibody (ED1; Chemicon, Temecula, CA, USA) diluted 1:300. The incubated sections were washed in PBS and incubated in biotinylated anti-mouse IgG antibody diluted 1:200 (Vector Laboratories, Burlingame, CA, USA) for 60 min at room temperature, and then washed in PBS and incubated for 30 min in avidin–biotin–peroxidase complex (ABC; Vector Laboratories). Finally, the sections were washed in PBS and developed in 0.02% 3,3'-diaminobenzidine dihydrochloride (DAB). After staining, tissue sections were coverslipped and examined with a light microscope. The investigator counting the ED1-IR cells was blind to experimental conditions. ED1-IR cells were analyzed in sections from the central one-third of the DRG. The total number of ED1-IR cells in each whole section was counted using imaging analysis software. Three randomly selected sections were examined per animal, and the mean for each animal was obtained. Results are expressed as the mean $\pm$ standard error for each experimental group.

Double immunofluorescence

Six micrometre-thick paraffin sections were prepared at days 3 and 7 as described above, and pretreated with 0.1% trypsin at 37°C for 20 min to enhance immunoreactivity. After blocking with 2% gelatin, double-label immunofluorescence was performed by incubating sections with primary antibody including goat anti-TNF (1:50) (R&D Systems Inc., Minneapolis, MN, USA) and ED1 (1:300) (Chemicon International) overnight at 4°C. The slides were then incubated for 1 h with a secondary antibody labeled with Alexa Fluor 488 (1:200) (green) or Alexa Fluor 555 (1:200) (red) (Molecular Probes, Carlsbad, CA, USA). Fluorescence staining was examined using an Olympus Optical BX50 fluorescence microscope

equipped with Axio Vision imaging software (Carl Zeiss, Göttingen, Germany).

Behavioral testing

In the saline, etanercept, and sham groups, 12 rats each were used to examine thresholds of withdrawal to mechanical and thermal stimulation at 1 day before surgery as a baseline, and 3, 7, 14, 21, and 28 days after surgery. For measurement of mechanical withdrawal thresholds, rats were placed in a transparent plastic box with a wire netting floor and allowed to acclimate for 15 min. The mid-plantar surface of the left hindpaw was stimulated using von Frey filaments capable of exerting bending forces of 0.7, 1.2, 2.0, 3.6, 5.5, 8.5, 15.1, and 28.8 g. We performed one trial for each filament using an up-down method as described in our previous report [38]. In total, six or more trials were performed for each rat. The force of stimulation that caused a brisk withdrawal in 50% of trials was calculated using Chaplan's method [3]. Results were expressed as the mean $\pm$ standard error (SE) of 50% withdrawal threshold.

For investigation of nociceptive responses to thermal stimulation, the rats were placed in a clear plastic chamber with a glass floor and were allowed to acclimate for 10 min before testing. A radiant heat source (Ugo Basille, Italy) was positioned under the glass floor directly beneath the hindpaw. The time of foot withdrawal upon thermal stimulation was measured as described in Hargreaves' report [8]. The hindpaw on each side was alternately tested at interstimulus intervals of 10 min, twice for each hindpaw. A difference score (DS) was calculated by subtracting the average latency of the control side from the average latency of the operated side [8]. Results are expressed as the mean $\pm$ SE of DS.

Statistical analysis

The Kruskal–Wallis test was used, followed by Mann–Whitney's U test with Bonferroni correction, with p values <0.05 considered significant.

Results

Expression of ED1-IR cells (Fig. 2a, b)

At day 3, there were no significant differences among the three groups. However, at day 7, marked increase in the number of ED1-IR cells was observed in the saline group, with a few ED1-IR cells observed in the sham group. At day 7, the number of ED1-IR cells was higher in the etanercept group than in the sham group ($p < 0.05$), but

Fig. 2 A Photomicrographs demonstrating ED1-IR cells in DRG at day 7. *a* Saline group, *b* sham group, and *c* etanercept group. These sections are transverse sections from one third of the central portion in DRG. In the saline group, many ED1-IR cells were observed in the DRG tissue, while only a few ED1-IR cells were observed in the sham and etanercept groups. Scale bar 50 μ m.

B Graph of numbers of ED1-IR cells in DRG. Results are the mean $\pm$ standard error of number of ED1-IR cells. At day 3, there were no significant differences in number of ED1-IR cells among the three groups. At day 7, marked increase in number of ED1-IR cells was observed in the saline group compared with the sham and etanercept groups ($p < 0.05$)

significantly less than that in the saline group ($p < 0.05$). These findings suggested that compression of the DRG induced increase in number of ED1-IR cells in the compressed DRG which was reduced by etanercept.

Double immunofluorescence for ED1 and TNF α (Fig. 3)

Photomicrograph of DRG at day 7 demonstrating immunoreactivity for ED1 (green) and TNF α (red) were presented. In the saline group, both neurons and the interneuronal regions exhibited immunoreactivity for TNF α , and co-localization for ED1 was observed. On the other hand, the sham and etanercept groups exhibited less

immunoreactivity for TNF α than the saline group. There were few ED1-IR cells in the sham and etanercept groups. At day 3, there were few ED1-IR cells exhibiting co-localization for TNF in each group (data not shown). These findings suggested that DRG compression induced increase in ED1-IR cells co-localized with TNF α which was inhibited by etanercept.

Mechanical withdrawal threshold (Fig. 4)

In the saline group, threshold decreased after DRG compression, and significant differences compared with the sham group were observed at days 7, 21, and 28. In the etanercept group, significant differences compared with

Fig. 3 Photomicrograph of DRG at day 7 demonstrating immunoreactivity for ED1 (green) and TNF α (red). In the saline group, some ED1-IR cells exhibited co-localization of staining for TNF α (triangles), with few ED1-IR cells in the sham and etanercept groups. Scale bar 50 μ m

the sham group were observed only at day 28, while the threshold decreased after operation. However, the etanercept group exhibited significant increase in threshold compared with the saline group at day 7 ($p < 0.05$). These findings indicated that etanercept attenuated the pain behavior induced by DRG compression.

Fig. 4 Time course of change in mechanical sensitivity. Results are the mean $\pm$ standard error of 50% withdrawal threshold. There were significant differences in threshold between the saline and sham groups at days 7, 21, and 28, and between the etanercept and sham groups at day 28 alone ($*p < 0.05$). The etanercept group exhibited significant differences in threshold from the saline group at day 7 ($**p < 0.05$). These findings suggested that DRG compression induced mechanical hypersensitivity from day 7 to day 28 which was attenuated by etanercept at day 7. The effect of etanercept appeared to disappear at day 28

Thermal withdrawal threshold (Fig. 5)

The difference score (DS) in the saline group was decreased after operation from day 3 to day 14, while the sham and etanercept groups exhibited no marked changes in DS from the baseline of zero. The saline group exhibited significant decrease in DS compared with the sham group as well as the etanercept group at day 7 ($p < 0.05$). These findings suggested that DRG compression induced hypersensitivity to heat stimulation that was attenuated by etanercept.

Discussion

Our findings showed that compression of the DRG induced increase in number of ED1-IR cells in the compressed DRG and expression of TNF α . In addition, etanercept, when applied directly to the DRG, attenuated the pain-related behaviors induced by DRG compression and inhibited the increase in ED1-IR cells. One previous study showed that in rats with chronic constriction injury (CCI), endoneurial TNF was increased within 12 hours after surgery [7]. In addition, several studies showed that anti-TNF α therapy for animals with nerve injury including DRG compression was effective only in the early stage of pain-related behavior [9, 30, 31]. These findings suggest that TNF α is related to the initiation of neuropathic pain. TNF α thus also appeared to be important in initiation of behavioral changes in our

Fig. 5 Time course of change in difference score (DS) as a measure of thermal latency. Results are the mean $\pm$ standard error of response time. In the saline group, DS decreased after DRG compression and was minimum at day 7 after surgery, while DS in the sham group did not decrease. There were significant differences between the saline group and sham group at day 7. On the other hand, in the etanercept group, DS did not significantly decrease during the observation period. There was a significant difference in DS between the etanercept and saline groups at day 7 (* $p < 0.05$). These findings suggested that thermal hypersensitivity was induced by DRG compression at day 7, which was attenuated by etanercept

animal model, and etanercept appeared to inhibit behavioral changes after DRG compression. However, in our model, development of behavioral changes was slower than in other neuropathic pain models. About one week is required to reach maximum effect in our model. Therefore, the time course of change in TNF α appeared to be slower than in other neuropathic pain models. This could explain why the effect of etanercept was observed only 7 days postoperatively. Interestingly, mechanical hypersensitivity tended to be reduced until day 21 by preventive application of etanercept. When etanercept is administered to humans via subcutaneous injection, its half-life is 4–5 days. However, the pharmacokinetics of etanercept applied via local injection in the rat have not been reported. The duration of therapeutic effect may have extended beyond the time of clearance of etanercept in this study.

Peripheral nerve injury induces inflammatory responses such as infiltration of inflammatory cells into the injured nerve tissue. In our study, the finding of increase in macrophages in the compressed DRG indicated that inflammatory response occurred after compression of the DRG, and that etanercept inhibited the inflammatory cascade in the compressed DRG by blocking TNF α . In addition, macrophages have other important functions such as phagocytosis. WLD mice, in which delayed Wallerian degeneration is observed after peripheral nerve injury, exhibit inhibition of neuropathic pain and macrophage infiltration [22]. Mice with knockout of chemokine

receptor 2 (CCR2), an important factor inducing infiltration of macrophages, exhibited no neuropathic pain after sciatic nerve ligation [1]. These previous findings suggest that macrophages have important functions in the mechanisms of neuropathic pain. There might thus be a relationship between reduction of infiltration of macrophages into the compressed DRG and improvement of pain-related behaviors by etanercept.

Previous basic research has shown that TNF α is closely related to the pain induced by nucleus pulposus [14, 19, 24–27, 29, 35], and anti-TNF α therapy has correspondingly been focused upon as a new treatment for lumbar disc herniation. The findings of the present study indicate that TNF α is also related to the pain induced by mechanical compression of the DRG. This may support the use of anti-TNF α therapy for the sciatica induced by lumbar disc herniation and lumbar spinal canal stenosis.

Regarding clinical use of anti-TNF α therapy, some groups have reported the efficacy of etanercept in treating low back pain and sciatica [4, 36]. Side-effects of these anti-TNF therapies are a significant problem, although no side-effects were reported [4, 36]. Not only infections due to immunosuppression but also demyelination could be a severe problem in radiculopathy with motor dysfunction. However, one experimental study showed that etanercept enhanced axonal regeneration in injured sciatic nerve [15]. Therefore, another study using different animal models which shows clear motor dysfunction is needed to examine the effect of etanercept on demyelination. Unsolved problems also remain concerning the route and timing of application of etanercept, as well as its dosage and selection of patients for treatment with it. Our findings showed that direct application of etanercept attenuated pain-related behavior and inflammatory reaction in DRG compressed rat. Therefore, direct application of etanercept to the nerve, such as nerve root infiltration, might be effective clinically. One clinical study indicated that transforaminal injection of etanercept could reduce sciatica [4]. In addition, the effective dosage with direct application is expected to be lower than that for subcutaneous injection, and this could reduce side effects of etanercept. In future studies, comparison of subcutaneous and direct application is needed.

In conclusion, etanercept, a soluble TNF α receptor, attenuated the pain-related behavior induced by DRG compression and inhibited increase in number of macrophages in compressed DRG. These findings suggest that TNF inhibition might not only modulate nucleus pulposus-induced effects but also the mechanical effects in sciatica induced by lumbar disc herniation. This suggests the usefulness of anti-TNF α therapy as an option for conservative treatment of the sciatica induced by lumbar disc herniation and spinal canal stenosis.

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