Original Article

Blockade of IL-6 receptor accelerates nerve regeneration in experimental sciatic nerve crush injury

**Miho Suzuki*, Hiroto Yoshida, Naohiko Hayakawa,
and Yoshihiro Matsumoto**

Product Research Department, Chugai Pharmaceutical Co., Ltd.

IL-6 is known as a multifunctional cytokine regulating immune response and inflammation. Many studies have shown that IL-6 affects the nervous system. However, the involvement of IL-6 in the nervous system, particularly in the peripheral nervous system (PNS), remains incompletely understood. Here, we assessed the effect of rat anti-mouse IL-6 receptor monoclonal antibody (MR16-1) in the treatment of experimental sciatic nerve crush injury in mice. The mice were given a single intraperitoneal injection (2 mg/head) of MR16-1 or Rat IgG as a control antibody the day before nerve crush injury. Treatment with MR16-1 significantly promoted sciatic functional recovery, and histological remyelination was greater than that by Rat IgG treatment. Furthermore, IL-6 expression was confirmed in the nerve after nerve crush injury in the Rat IgG group, but not in the MR16-1 group. MR16-1 also decreased the number of infiltrating macrophages. Additionally, IL-6 decreased the expression of nerve growth factor (NGF) mRNA in cultured primary mouse Schwann cells, and this decrease in NGF expression by IL-6 was reversed with MR16-1. These findings suggest that MR16-1 may inhibit the infiltration of macrophages and the decrease of NGF in Schwann cells, resulting in the acceleration of nerve regeneration.

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*Correspondence should be addressed to:

Miho Suzuki, Product Research Department, Chugai Pharmaceutical Co. Ltd., 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan. Phone: +81-550-87-6379, Fax: +81-550-87-6782, E-mail: suzuki.miho@chugai-pharm.co.jp

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Introduction

The peripheral nervous system (PNS) is composed of neurons and glial cells, as in the case with the central nervous system (CNS). The glial cells that sheathe axons in the PNS are known as Schwann cells. If nerve fibers in the PNS become damaged, a self-destructive process called

Wallerian degeneration (WD) is initiated as follows¹⁾: (1) the axon and myelin sheath distal to the injury site disintegrate; (2) a variety of pro-inflammatory cytokines are produced; (3) Schwann cells are activated and proliferate; and (4) infiltration of inflammatory cells is promoted. These steps are not yet fully understood. After WD, neurotrophic

factors such as nerve growth factor (NGF) are produced and axons elongate, which is the first step in nerve regeneration²⁾.

IL-6, which is produced by a variety of cells including monocytes, fibroblasts, and endothelial cells, is a multi-functional cytokine that regulates the immune response, hemopoiesis, the acute-phase response, and inflammation^{3,4)}. With respect to the role of IL-6 in the nervous system, many reports have been published on its effects on nerve lesions, and some studies suggest that the inhibition of IL-6 signaling acts as a positive regulator of nerve regeneration⁵⁻⁷⁾. The main reason is thought to be that IL-6 produced in response to nerve injury promotes the chemotaxis of inflammatory cells such as macrophages or that inflammatory cells infiltrating as a result of nerve injury produce IL-6, resulting in delayed nerve regeneration. On the other hand, however, much conflicting evidence has also been reported that IL-6 acts as a neurotrophic factor⁸⁻¹²⁾. Therefore, the involvement of IL-6 in the nervous system is controversial.

In recent research to investigate the PNS, the sciatic nerve crush model has been used as a model of peripheral nerve regeneration following transient WD¹³⁻¹⁵⁾. In this model, it has been reported that the expression of IL-6 is up-regulated the day after nerve crush injury¹⁶⁾. In studies using IL-6 knockout mice, it has been reported that IL-6 does not have a positive effect on functional recovery¹⁷⁾ but that IL-6 is essential for modulation of sensory functions⁸⁾. However, not enough is known about the effect of IL-6 on the PNS. In this study, we examined the influence of anti-IL-6 receptor antibody in mice with sciatic nerve crush injury.

Materials and Methods

1) Reagents

Recombinant mouse IL-6 and IL-6 receptor (IL-6R) were purchased from R&D Systems (Minneapolis, MN, USA). Rat anti-mouse IL-6R monoclonal antibody (MR16-1) for *in vivo* study was prepared in our laboratories¹⁸⁾. Rat IgG (control) was purchased from MP Biomedicals (Aurora, OH, USA). Anti-mouse IL-6R antibody (Clone: D7715A7) for *in vitro* study was purchased from Biolegend (San Diego, CA, USA).

2) Animals

Male C57BL/6J mice (7 weeks old) were purchased from

Charles River Japan (Yokohama, Japan). Mice were specific pathogen free and were kept in cages in a room maintained at 20-26°C and 35-75% relative humidity. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co, Ltd.

3) Sciatic nerve crush

Mice were anesthetized with isoflurane. The sciatic nerves were exposed through an incision in the mid-thigh of each hind limb and crushed by maximally clamping the nerve with a smooth-jawed micro-forceps. After nerve crush injury, the surgical incisions on both lateral aspects were closed.

On the day before nerve crush injury, mice were intraperitoneally injected with a single dose of MR16-1 (2 mg/head) or with the same volume and concentration of Rat IgG.

4) Walking track analysis

To examine functional motor recovery, walking track analysis was performed on mice in the three different groups (sham-operated, Rat IgG, and MR16-1 groups) before nerve crush injury and at 7, 8, 10, 11, 12, 13, 15, and 17 days after nerve crush injury, as previously described¹³⁻¹⁵⁾. Briefly, paw-prints of mice were recorded by painting the hind paws of the mice with ink and having them walk along a 6 cm x 40 cm corridor lined with white paper. After the paw-prints were scanned, the toe spread and paw length were measured. The sciatic functional index (SFI) was calculated according to the following formula¹⁹⁾:

$$SFI = \frac{118.9(ETS-NTS)}{NTS} - \frac{51.2(EPL-NPL)}{NPL} - 7.5$$

where *ETS* is the experimental toe spread, *NTS* is the normal toe spread, *EPL* is the experimental paw length, and *NPL* is the normal paw length. A value of *SFI* close to zero indicates normal nerve function, and a value around -100 implies total impairment.

5) Quantitation of degenerating and myelinated axons

Histological samples were taken on the day after nerve crush injury and stained with toluidine blue in an alkaline solution. Axons wrapped to a greater or lesser extent with myelin sheaths were counted as myelinated. White and clearly stained myelin profiles were classified as myelinated

axons, and irregularly and darkly stained myelin profiles were classified as degenerating myelin sheaths.

6) Immunohistochemistry

Histological samples were taken on the day after nerve crush injury. Sciatic nerves with muscle were dissected and mounted in OCT compound and frozen in hexane cooled in dry ice-acetone. Sections from the frozen samples were stained with anti-IL-6 antibody (Invitrogen, Carlsbad, CA, USA). Samples were also stained with anti-F4/80 antibody (Serotec, Düsseldorf, Germany) after the sciatic nerves had been dissected and fixed in 10% formalin. All samples were examined under a fluorescence microscope.

7) Isolation of Schwann cells

Schwann cells from the sciatic nerves of adult male C57BL/6J mice were isolated according to a modification of a previously reported method⁽²⁰⁾. Dissected sciatic nerves were cultured in 24-well plates containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. They were transferred into new plates once a week, and the medium was changed twice a week. After culturing for 2 weeks, the pieces were placed in DMEM containing 1.25 U/mL dispase (Roche, Mannheim, Germany) and 0.05% collagenase (Wako Pure Chemical Industries, Osaka, Japan), and incubated overnight at 37°C in a 5% CO₂ atmosphere. The next day, they were dispersed by gentle pipetting and washed 2 or 3 times with DMEM. The suspension was placed in 100 mm culture dishes; 1 h later, nonadherent cells were collected and placed in new dishes. This procedure was repeated 2 more times, and the obtained nonadherent cells were seeded in poly-L-lysine-coated 100 mm culture dishes containing DMEM. On the following day, cytarabine (Wako) was added to a final concentration of 10⁻⁵ M, and cells were cultured for another 3 days. The remaining cells were grown in DMEM containing forskolin (2 μM) (Sigma Aldrich, St. Louis, MO, USA) and bovine pituitary extracts (10 μg/mL) (Cosmo Bio, Tokyo, Japan). The medium was changed twice a week for the following experiments.

8) Immunostaining of cultured Schwann cells

The cells were cultured in slide flasks overnight and fixed with 4% paraformaldehyde for 20 min at room temperature. Fixed cells were washed for 10 min in PBS, and then

permeabilised using 0.2% (v/v) Triton X-100 for 20 min. The cells were washed twice for 10 min in PBS, and PBS containing 5% (v/v) goat serum (Sigma Aldrich) was added for 1 h at room temperature. Polyclonal rabbit anti-S100 antibody (1:400; DakoCytomation, Carpinteria, CA, USA) in PBS containing 1% (v/v) goat serum was added and cells were incubated at 4°C overnight. Cells were washed 3 times for 10 min in PBS, and Dylight 488 anti-rabbit IgG (1:150; Vector Laboratories, Burlingame, CA, USA) in PBS containing 1% (v/v) goat serum was added for 1 h at room temperature. The cells were washed 3 times for 10 min in PBS, and slides were mounted using Prolong Gold antifade reagent (Invitrogen, Eugene, OR, USA). Slides were examined under a fluorescence microscope.

9) Cell culture

Mouse Schwann cells (1 × 10⁵ cells/12 wells) were cultured with 100 ng/mL mouse IL-6 + 100 ng/mL mouse sIL-6R and 100 μg/mL anti-mouse IL-6R antibody (Clone: D7715A7) for 24 h. After culturing, cell lysates were collected.

10) Quantitative real-time PCR

Total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA, USA). Synthesis of cDNA was performed using an Omniscript RT kit (Qiagen) with random 9-mer primers (Takara Bio, Shiga, Japan) according to the manufacturer's protocol.

Quantitative real-time PCR was performed by running a TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) targeting mouse NGF and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) on an ABI PRISM 7500 system (Applied Biosystems) according to the manufacturer's protocol.

Results

1) Functional recovery following crushed sciatic nerve

We used walking track analysis to examine the effect of MR16-1 on functional recovery after nerve crush injury. The *SFI* value is an important parameter for evaluating functional recovery in this analysis. The *SFI* value is a value of toe spread and paw length standardized by a control value (in this case, toe spread and paw length before nerve crush injury): the higher the value, the closer that sciatic function is to normal; the lower the value, the closer that sciatic function is to significant dysfunction. Figure 1 shows

Fig.1 Effect of MR16-1 on functional recovery following crushed sciatic nerve

(A) The sciatic function index (SFI) values of mice after treatment with Rat IgG (2 mg/head) or MR16-1 (2 mg/head) before and after nerve crush injury. (B) Representative footprints of sham-operated, Rat IgG, and MR16-1 groups at 10 days after nerve crush injury. All data are represented as means + SD. Statistical significance was analyzed by unpaired *t*-test ($n = 9-10$, $*p < 0.05$ vs. Rat IgG group).

the changes in the SFI value from 7 days to 17 days after nerve crush injury and representative footprints 10 days after nerve crush injury. There were significant differences in SFI value between the Rat IgG group and the MR16-1 group at 8 to 11 days after nerve crush injury (Fig.1A). The main difference between these groups was the value of toe spread (Fig.1B). These results show that the MR16-1 group exhibited better functional recovery than Rat IgG group.

2) Remyelination in crushed sciatic nerve

Next, we examined by toluidine blue staining whether MR16-1 affected nerve regeneration at the morphological level after nerve crush injury. Myelinated axons stained with toluidine blue were assessed and counted by light microscopic imaging at 5, 10, 15, and 20 days after nerve crush injury. At 5 days after nerve crush injury, demyelination was observed distal to the lesion in both the Rat IgG

Fig.2 Effect of MR16-1 on remyelination in crushed sciatic nerve

(A) Toluidine-blue-stained sections of sciatic nerves a few millimeters distal to the lesion in representative mice from the Rat IgG and MR16-1 groups at 5, 10, 15, and 20 days after nerve crush injury. The arrows indicate degeneration and arrowheads indicate regeneration of myelin. Scale bar, 50 μ m. (B) Densities of myelinated axons in profiles of Rat IgG and MR16-1 groups at 5, 10, 15, and 20 days after nerve crush injury. All data are represented as means + SD. Statistical significance was analyzed by unpaired *t*-test ($n = 5$, $*p < 0.05$ vs. Rat IgG group).

group and the MR16-1 group, and this nerve degeneration declined over time (Fig.2A, arrows). Additionally, remyelination was confirmed in the MR16-1 group as early as 5

Fig.3 Expression of IL-6 and the infiltration of macrophages in crushed sciatic nerve

IL-6 immunostaining in sciatic nerves from representative mice of the Rat IgG group (A) and the MR16-1 group (B), and F4/80 immunostaining in sciatic nerves from representative mice of the Rat IgG group (C) and the MR16-1 group (D) the day after nerve crush injury. The areas indicated by squares are shown at higher magnification. The arrowheads indicate positive cells. Scale bar, 50 μ m.

days after nerve crush injury, and at day 20 there was not much difference between the sham-operated group and MR16-1 group (Fig.2A, arrowheads). Furthermore, the number of myelinated axons gradually increased in both the Rat IgG group and the MR16-1 group as the days went on. The number of myelinated axons in the MR16-1 group

Fig.4 Expression of NGF in Schwann cells treated with IL-6

(A)Primary Schwann cells from adult murine sciatic nerves immunostained with S100. (B)Expressions of NGF in Schwann cells treated with IL-6 (100 ng/mL) + sIL-6R (100 ng/mL) and anti-mouse IL-6R antibody (Clone: D7715A7) (100 μ g/mL) for 24 h. All data are represented as means + SD. Statistical significance was analyzed by unpaired t-test ($n = 3$, $*p < 0.05$ vs. IL-6+sIL-6R).

was significantly larger than in the Rat IgG group at 5 to 15 days after nerve crush injury, although at day 20 numbers were similar in both groups (Fig.2B).

3)Expression of IL-6 and the infiltration of macrophages following crushed sciatic nerve

To determine what led to this nerve recovery in the MR16-1 group, we used immunostaining to examine the expression of IL-6 and the infiltration of macrophages in the crushed sciatic nerve. In the Rat IgG group, high expression of IL-6 (Fig.3A) and many F4/80 positive cells (Fig. 3C) were observed the day after nerve crush injury. On the other hand, in the MR16-1 group, expression of IL-6 was not observed (Fig.3B) and fewer F4/80 positive cells were observed (Fig.3D). These results indicate that MR16-1 attenuated the infiltration of macrophages following nerve crush injury.

4) Expression of NGF in Schwann cells treated with IL-6

To demonstrate the purity of the murine Schwann cells, cells were analyzed by immunostaining with S100 protein recognizing Schwann cell-specific protein. All cells were S100-positive (Fig.4A). Next, we examined the effect of IL-6 on the expression of NGF mRNA in murine Schwann cells. Cells were cultured with IL-6 (100 ng/mL) + sIL-6R (100 ng/mL) and anti-IL-6R antibody (Clone: D7715A7) (10 μ g/mL) for 24 h. Non-stimulated cells spontaneously expressed NGF. IL-6 significantly suppressed the expression of NGF mRNA, and anti-IL-6R antibody recovered this decreased expression (Fig.4B).

Discussion

In this study, we confirmed that (1)MR16-1 improved functional recovery from nerve injury; (2)MR16-1 promoted the remyelination of axons after nerve crush injury; (3)IL-6 was expressed shortly after nerve crush injury; (4)MR16-1 attenuated the infiltration of macrophages following nerve crush injury; and (5)IL-6 significantly suppressed the expression of NGF mRNA in primary Schwann cells, and anti-IL-6R antibody recovered this decreased expression. These data suggest that the blockade of IL-6 receptor accelerates nerve regeneration by suppressing infiltration of macrophages and promoting NGF secretion from Schwann cells in experimental sciatic nerve injury.

Footprint analysis is a commonly used method to evaluate functional recovery in the sciatic nerve crush model. We showed that MR16-1 accelerated functional recovery of injured nerves (Fig.1). In this method, although a low *SFI* value was obtained in mice with a dysfunctional sciatic nerve, we started *SFI* assessment from 7 days after nerve crush injury because the nerve dysfunction was too severe to obtain footprints. Although there was no significant difference in functional recovery between the Rat IgG group and the MR16-1 group at 7 days after nerve crush injury, which was the earliest point examined in this study, remyelination in the MR16-1 group was significantly better than in the Rat IgG group at 5 days after nerve crush injury (Fig.2). Therefore, we consider that MR16-1 must have at least some kind of influence on nerve regeneration by 5 days after nerve crush injury.

Many researchers have shown that IL-6 expression increases soon after nerve damage in models of transient peripheral nerve injury^{16, 21}. We also confirmed the increased expression of IL-6 in the nerve tissue of mice in

the Rat IgG group the day after nerve crush injury (Fig.3A). It has been reported that IL-6 is produced by Schwann cells and macrophages^{16, 22-25}. However, in our study, IL-6 expression was not confirmed in nerve tissue of mice in the MR16-1 group (Fig.3B). Some studies have indicated that Schwann cells can initiate the process of WD by releasing proinflammatory cytokines such as IL-6^{22, 26}. However, our results indicate the high possibility that the IL-6 induced after nerve crush injury is not secreted from Schwann cells.

It has been thought that the infiltration of inflammatory cells such as macrophages into crush lesions is important for nerve recovery after injury, because these cells clear degenerating axons and myelin after injury and help regenerate the nerve²⁷⁻²⁹. On the other hand, it has also been reported that these inflammatory cells inhibit nerve regeneration through the secretion of inflammatory cytokines^{5, 30, 31}. In our study, a number of macrophages were confirmed in the nerve tissue of mice in the Rat IgG group (Fig.3C). However, very few macrophages were confirmed in the nerve tissue of mice in the MR16-1 group (Fig.3D). At the least, these results indicate the low probability of infiltrating macrophages the day after injury contributing to nerve recovery. Although moderate numbers of macrophages might contribute to nerve regeneration by phagocytosis, too many macrophages might induce an excessive inflammatory response, resulting in the opposite effect. We also confirmed that the functional recovery tended to be faster in IL-6-deficient mice (data not shown); however, this recovery was modest at best. Perhaps it is preferable to block IL-6 signaling only soon after nerve injury because macrophages infiltrating at a later stage than the day after injury might be involved in the recovery of the nerve. In any case, in our model, it is thought that macrophages infiltrating on the day after injury inhibited the nerve recovery through the production of IL-6.

Our study indicated that macrophages that infiltrate into the crush lesion the day after injury produce IL-6 and delay nerve regeneration. To explore how IL-6 affects nerve regeneration, we examined the effect of IL-6 on the expression of NGF which is an essential factor in nerve regeneration³². We cultured primary Schwann cells isolated from the sciatic nerve of adult mice and found that the expression of NGF in Schwann cells was decreased by IL-6 stimulation (Fig.4). It has been reported that the expression of not only IL-6 but also NGF is increased in

nerve tissue after injury³³⁾ and that IL-6 acts as a neurotrophic factor^{34, 35)}. Therefore, some say that the increased production of IL-6 after injury is important for nerve recovery. However, our data indicate that IL-6 is a negative factor for nerve recovery. IL-6 might interfere with the secretion of NGF from Schwann cells following nerve crush injury.

In conclusion, blockade of IL-6 receptor accelerated nerve regeneration in experimental sciatic nerve crush injury by suppressing the infiltration of macrophages that produce IL-6. Our data suggest that blockade of IL-6 receptor might be useful for treating peripheral nerve injury.

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