Blockade of interleukin-6 receptor suppresses inflammatory reaction and facilitates functional recovery following olfactory system injury

Masayoshi Kobayashi [*,1], Kengo Tamari [1], Tomotaka Miyamura [1], Kazuhiko Takeuchi

Department of Otorhinolaryngology-Head and Neck Surgery, Mie University Graduate School of Medicine, Japan

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ABSTRACT

We previously reported that anti-inflammatory treatment with steroids improves recovery outcome in an olfactory nerve injury model. Clinically, however, steroid administration is not recommended in the acute phase of head injury because of concerns regarding side effects and no evidence of its efficacy. Recently, it has been reported that interleukin-6 (IL-6) plays an important role in the inflammatory reaction. The present study investigates if anti-IL-6 receptor (IL-6R) antibody can facilitate functional recovery in the olfactory system following injury. Rat anti-mouse IL-6R antibody (MR16-1) was intraperitoneally injected to severe olfactory nerve injury model mice immediately after the nerve transection (NTx). Histological assessment of recovery within the olfactory bulb was made at 5–70 days. X-gal staining labeled the degenerating and regenerating olfactory nerve fibers and immunohistochemical staining detected the presence of reactive astrocytes and macrophages/microglia. MR16-1-injected animals showed significantly smaller areas of injury-associated tissue, fewer astrocytes and macrophages/microglia, and an increase in regenerating nerve fibers. Olfactory function assessments using both an olfactory avoidance behavioral test and evoked potential testing showed improved functional recovery in MR16-1-injected mice. These findings suggest that blockade of IL-6R could provide a new therapeutic strategy for the treatment of olfactory dysfunction following head injuries.

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1. Introduction

Olfactory dysfunction is relatively common following head trauma. Early estimates of the incidence of posttraumatic olfactory dysfunction based on 32 clinical reports average around 8% (Hendriks, 1988). Recently, new reports suggest the incidence is higher averaging 11–22% (De Kruijk et al., 2003; Haxel et al., 2008; Sigurdardottir et al., 2010). Since loss of olfactory function lowers patients' quality of life and can be life threatening due to the inability to detect hazardous events such as fire, gas leak, and spoiled food intake (Miwa et al., 2001; Santos et al., 2004), therapeutic management of patients aimed at improving olfactory dysfunction is an important clinical objective.

Head trauma can cause olfactory dysfunction by overextension, distortion and tearing of the olfactory nerves and contusions of the olfactory bulbs and orbitofrontal regions of the brain (Costanzo et al., 2012). Although the olfactory system has a remarkable capacity for neural regeneration and recovery following injury, olfactory function does not recover in many cases of severe head injury.

The prognosis for recovery from olfactory dysfunction after head trauma is only 10–38% (Sumner, 1964; Zusho, 1982; Costanzo and Becker, 1986; Jimenez et al., 1997; London et al., 2008), while that caused by inflammatory diseases as chronic rhinosinusitis and allergic rhinitis are reported to have a relatively better prognosis, with recovery rates of up to 68–86% (Delank and Stoll, 1998; Kobayashi et al., 2005; Miwa et al., 2005). We previously demonstrated using olfactory nerve injury model in mice that recovery in the olfactory system depends on the severity of the damage. We found that anti-inflammatory treatment with steroids during the acute phase of injury is effective in suppressing the inflammatory reaction and local glial scar formation and improves recovery outcome after olfactory nerve transection (NTx) (Kobayashi and Costanzo, 2009). In clinical practice, however, steroids are not typically used for the treatment of head injury patients since several studies reported that steroids do not have a significant efficacy on morbidity and mortality in patients with severe head injury and there are concerns that steroids may cause serious side effects such as hypertension, hyperglycemia, infection, bone necrosis and psychosis (Cooper et al., 1979; Braakman et al., 1983; Dearden et al., 1986).

Interleukin-6 (IL-6) has proven to play an important role in regulating the inflammatory reaction and the anti-IL-6 receptor (IL-6R) antibody has been reported to be effective inhibitor of the inflammatory reaction by preventing IL-6 from combining with

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* Corresponding author at: 2-174 Edobashi, Tsu, Mie 514-8507, Japan.
Tel.: +81 59 231 5028; fax: +81 59 231 5218.
E-mail address: m-koba@doc.medic.mie-u.ac.jp (M. Kobayashi).
[1] These authors contributed equally to this work.

the IL-6 receptor (Taga and Kishimoto, 1997; Rose-John et al., 2006). Recently anti-human IL-6R antibody (tocilizumab) has been used instead of steroids for the treatment of refractory inflammatory diseases such as rheumatoid arthritis, Castleman's disease and juvenile idiopathic arthritis (Nishimoto et al., 2000, 2004; Yokota et al., 2005). For the central nervous system, a recent study reported that administration of anti-IL-6R antibody suppresses glial scar formation and ameliorates functional recovery of experimentally injured spinal cord in mice (Okada et al., 2004). The present study was designed to investigate if therapeutic intervention using anti–IL-6R antibody is effective on recovery outcomes in olfactory system following injury in mice. In this study, we used histological techniques to examine recovery outcome by following the amount of injury-associated tissue (glial scar), reactive astrocytes (GFAP) and macrophages/microglia (CD68) and the degree of degeneration and regeneration of olfactory nerve fibers. We also administered an olfactory function test using avoidance conditioning behavior to odorants and electrophysiological recording of field potential responses to electrical stimulation of the olfactory mucosa to determine if morphological recovery would parallel functional recovery in the olfactory system following therapeutic intervention.

2. Materials and methods

2.1. Experimental animals

This study was performed using a transgenic strain of mice (OMP-tau-lacZ mice) obtained from the Jackson Laboratory (Bar Harbor, ME, USA). This strain is derived from C57BL/6 mice. In OMP-tau-lacZ mice, the gene sequence encoding the olfactory marker protein (OMP) has been replaced with a tau-lacZ reporter gene (Mombaerts et al., 1996). The OMP is expressed in all mature olfactory neurons (Farbman and Margolis, 1980) and the replacement with tau-lacZ reporter gene allows for the visualization of olfactory nerve fibers and their projections to olfactory bulb glomeruli.

2.2. Surgical procedures

We used both male and female OMP-tau-lacZ mice randomly. They are adult mice whose ages range from 20 to 36 weeks, weighing 32.0 ± 0.5 g ($n = 72$). They were anesthetized by intraperitoneal injection of sodium pentobarbital (80 mg/kg). After anesthesia was obtained, a frontal craniotomy was performed and the olfactory bulbs were exposed. The olfactory nerve transection (NTx) was performed between the olfactory bulb and cribriform plate using a curved rigid stainless steel blade (Fig. 1A). The use of a rigid blade resulted in a severe injury that included adjacent regions of the olfactory bulb and bony cribriform plate (Kobayashi and Costanzo, 2009). For histological assessments, the NTx was performed on the left side (injury side) of the animal while the right side (right olfactory bulb and nerves) remained intact and served as an internal control (Fig. 1B). For behavioral and electrophysiological studies, the NTx was performed bilaterally. After the NTx was complete, the skin incision was sutured and the animal closely monitored until it was awake and fully recovered from anesthesia. All protocols and surgical procedures for this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Mie University.

2.3. Anti-IL-6R antibody injection

To determine how IL-6 is associated with inflammatory reaction and nerve regeneration after injury, rat anti-mouse IL-6R monoclonal antibody, MR16-1 (Chugai Pharmaceutical, Tokyo, Japan), was administered to mice receiving the severe injury NTx surgery. MR16-1 was injected intraperitoneally just after the NTx at Day 0 (day of surgery). Low (2 mg) and high (4 mg) doses of MR16-1 were used to determine if there is a dose-dependent effect of the drug. For control animals, rat IgG (Jackson Immuno Research, USA; 2 mg) was also injected intraperitoneally. We referred to a previous study for administration of MR16-1 and control IgG (Okada et al., 2004). Data were collected from 6 mice for each of the 3 treatment groups and 4 recovery time points (Day 5, 14, 42, 70) within each group (total, $n = 72$).

2.4. Tissue preparation

At 5, 14, 42 and 70 days after the left olfactory NTx injury, mice were deeply anesthetized with sodium pentobarbital (90 mg/kg) and fixed by intracardiac perfusion using 4% paraformaldehyde in phosphate buffer following a saline rinse. The nasal cavity and anterior portion of the skull including olfactory bulbs were removed *en bloc* and postfixed by immersion in 4% paraformaldehyde for 1 h and then placed in a decalcification solution of 0.5 M EDTA (ethylenediaminetetraacetic acid) for 14 days. The tissue was cryoprotected with 30% sucrose for 2 days, then immersed in embedding compound, quickly frozen in −80 °C freezer and sectioned on a cryostat.

Serial horizontal sections along dorsum nasi were cut at 30 μm and mounted on glass slides.

2.5. X-gal staining

Tissue sections were washed at room temperature with buffer A [100 mM phosphate buffer (pH 7.4), 2 mM $MgCl_2$ and 5 mM EGTA (ethylene glycol tetraacetic acid)] once for 5 min and then a second time for 25 min. This was followed by two 5 min washes with buffer B [100 mM phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P40]. The blue X-gal reaction was generated by overnight exposure in the dark to buffer C (buffer B, with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml of X-gal). The X-gal reaction was stopped by two 5 min washes in phosphate buffer.

2.6. Measurement of injury-associated tissue and nerve recovery

After confirming the appearance of the blue X-gal reaction, tissue sections were counterstained using a 1% Neutral Red solution. Sections were examined and digitized using CCD photomicroscopy. Areas of injury-associated tissue, including inflammatory cells and glial scar tissue, were identified along with blue (X-gal) labeled olfactory nerve endings within the glomerular layer of the olfactory bulb (Fig. 1B).

The area of injury-associated tissue was outlined on digital images of tissue sections and quantified using ImageJ (ver.1.36, National Institute of Health [NIH]) software. The area (mm^2) of proliferating tissue observed between the cribriform plate and olfactory bulb (Fig. 1B) was measured in two representative horizontal sections (sections A and B) from each animal and averaged. Section A was selected to represent the dorsal level. At this particular level a large olfactory nerve bundle is observed passing from endoturbinate II through the cribriform plate to the olfactory bulb. Section B represented a more ventral level. At this level endoturbinate III attaches to the cribriform plate. The area measurements from NTx mice at each of the four recovery time points were used to compare the mean values for injury-associated tissue.

Levels of olfactory nerve degeneration and regeneration were assessed by comparing changes in the amount of blue X-gal staining in the glomerular layer on the left (NTx injury side) to that on the right (control) side. Horizontal olfactory bulb sections (Sections A and B) were also used to obtain measurements of: (1) the glomerular layer perimeter distance (G-P distance), a continuous line passing through the center of all the glomeruli within the bulb section, and (2) the total length of glomerular segments along the perimeter that were labeled with the blue X-gal stain (G-X-gal-distance). The ratio of the X-gal-stained distance (G-X-gal-distance) to the total perimeter of the glomerular layer (G-P distance) was obtained for both the NTx injury and control sides. Changes in the blue X-gal staining on the NTx injury-left side were expressed as percentage of the X-gal staining on the intact control side and were used to measure levels of olfactory nerve degeneration and regeneration within the olfactory bulb, as follows:

% olfactory nerve innervation to the glomerular layer

$$= \frac{\text{G-X-gal-distance of NTx side/G-P distance of NTx side}}{\text{G-X-gal-distance of control side/G-P distance of control side}} \times 100 \quad (\%)$$

2.7. Immunohistochemical assessments

Immunohistochemical staining for glial fibrillary acidic protein (GFAP) and cluster of differentiation (CD68) glycoprotein was performed on horizontal sections at four different time points following injury, Day 5, 14, 42 and 70. GFAP is constitutively produced by astrocytes. In the reactive glial response to CNS injury, hypertrophic reactive astrocytes increase their expression of GFAP (Silver and Miller, 2004). CD68 staining was used to measure injury-induced inflammatory changes at different time points after NTx injury. CD68 is a lysosomal membrane-associate glycoprotein that is expressed on the surface of histiocytes, cells that are part of the immune system, including macrophages, monocytes and microglia and play an important role in phagocytic activities.

After washing with phosphate-buffer saline (PBS) for 5 min, sections were processed by immersion for 1 min intervals in a series of alcohol solutions (70, 95, 100, 95, 70% ethanol). This was followed by three 5 min washes with 0.3% Triton X-100 in PBS. Sections were then incubated with 5% normal goat serum, 1% bovine serum albumin, 0.5% Triton X-100 in PBS for 30 min and reacted with one of the following primary antibodies: rabbit anti-mouse GFAP antibody (1:500, DAKO, USA) and rat anti-mouse CD68 antibody (1:100, AbD serotec, USA). These antibodies were visualized using Cy3-conjugated goat anti-rabbit IgG (1:100, GE, USA) and Alexa Fluor 488-conjugated goat anti-rat IgG (1:100, Invitrogen, USA) under fluorescent microscope, respectively. GFAP- and CD68-positive cells were counted in five different 0.01 mm^2 sampling areas located in the anterior (injured) region of the olfactory bulb (five areas: the anterior apex area, areas adjacent (medial and lateral) to the anterior apex area, and areas medial and lateral to the adjacent areas). The average number of GFAP and CD68-positive cells/0.01 mm^2 were then calculated for the NTx mice at each of the four recovery time points.

A

Stainless steel blade

OB

OE

CP

B

ET II

ON

GL

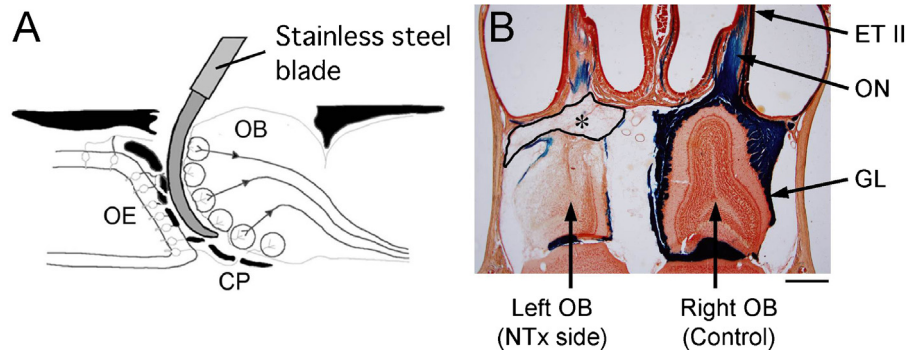Left OB (NTx side)   Right OB (Control)

Fig. 1. An experimental model of severe olfactory bulb deafferentation injury. (A) A curved rigid stainless steel blade is inserted between the cribriform plate and olfactory bulb resulting in transection of the olfactory nerves (NTx) and additional injury to the outer layers of the olfactory bulb as well as the cribriform plate. (B) A horizontal section through the nasal cavities and olfactory bulbs illustrating differences observed between the lesioned (left) and control (right) sides at 5 days after NTx injury. The area of injury-associated tissue (glial scar) formed between the cribriform plate and olfactory bulbs (enclosed by a black line, *) was measured and quantified using ImageJ software (ver.1.36, NIH, USA). The olfactory nerves and their projections to glomeruli are labeled using an X-gal staining method (blue color). CP, cribriform plate; GL, glomerular layer; OB, olfactory bulb; OE, olfactory epithelium; ON, olfactory nerve; ET II, endoturbinate II. Calibration bars in B = 500 μm.

2.8. Olfactory function test

To examine if olfactory function is restored after the olfactory NTx, a smell detection test that uses avoidance conditioning behavior to cycloheximide, was administered to mice before and after the NTx. This assay of smell detection ability is described in a previous report (Shiga et al., 2008). Briefly, cycloheximide has a peculiar odor and unpleasant taste for mice. Normal mice were first deprived of water for 48 h and then trained to avoid cycloheximide solution. Before the NTx, the mice were conditioned in two or more training sessions, each consisting of 10 trials. In each trial, the mouse was presented with a bottle of 0.01% cycloheximide solution and one with distilled water positioned on the left and right side of an animal cage. When the mouse licked the delivery tube of either bottle, the bottles were temporally withdrawn from view and presented again. The left and right positions of the two bottles were shifted according to the Gellermann series (cycloheximide bottle position: right (R)-left (L)-L-R-L-L-R-R-R-L). Mice were considered to have learned the smell of cycloheximide when they chose the distilled water bottle 10 consecutive times out of 10 trials (percent score: 100%). After the NTx surgery, the test was administered again every four days until the mouse regained its olfactory function (scored 10 out of 10 correct responses). When the animal showed 100% score again, we regarded that its olfactory function fully recovered. The test was continued until Day 100 and cut off if the mouse did not show 100% score on the day.

2.9. Electric field potential recording

To confirm that regenerated olfactory receptor cell axons were functionally reconnected to the olfactory bulb after the olfactory NTx, field potentials evoked by electrical stimulation of the olfactory mucosa were recorded in the olfactory bulb of mice that showed behavioral recovery in the cycloheximide olfactory function test. In this series of experiments, the olfactory nerves were sectioned bilaterally resulting in a complete loss of olfactory function. On the first recording day (after recovery was established by behavioral testing), the animal was anesthetized and the nasal bone was removed to expose the olfactory mucosa and a second craniotomy was performed to expose the olfactory bulb. The olfactory mucosa was electrically stimulated using a concentric circular needle electrode (200 μm in diameter) with a stimulus condition of 0.5 mA, 0.3 ms, and 1 Hz. Field potentials were recorded from a region of the ipsilateral olfactory bulb 1000 μm lateral to the midline, 6000 μm rostral to the bregma, and 1000 μm ventral to the surface. The recording microelectrode used in this experiment was a glass capillary pipette filled with 0.5 M KCl with a tip resistance of 3–6 MΩ. For precise evaluation of the effects of olfactory mucosa stimulation, in each case, olfactory mucosa stimulation-induced responses were averaged for 32 stimulus trials to improve the signal-to-noise ratio of the recordings. The magnitude of field potential was quantified by measuring the time integral of evoked field potential. Recordings were made within a day after olfactory function was determined to be fully restored by the behavioral olfactory function test. Mice that failed to show recovery in the behavioral olfactory function test were assessed for field potential responses on Day 100.

2.10. Statistical analysis

All numerical data obtained in the present experiments were expressed as means ± standard errors (SE). For statistical analysis of the data, the Mann–Whitney U-test was used to determine differences in average values between two groups. For three groups, the two-way ANOVA was used and *post hoc* comparisons were performed by the Bonferroni method. The chi-square (χ^2) test for independence was used to test for differences in ratio. Differences were regarded as significant when $p < 0.05$ for two group and $p < 0.0167$ for three group comparisons.

3. Results

3.1. Effects of anti-IL-6R antibody injection

To determine if anti-IL-6R antibody treatment can facilitate recovery of the olfactory nerves after NTx injury, MR16-1 was injected intraperitoneally in the severe injury model mice. Fig. 2 gives results of MR16-1 treatment compared to effects of control IgG. In X-gal stained tissues, blue glomeruli on the NTx side decreased at Day 5 and 14 after the NTx, indicating that degeneration of the olfactory nerves at the nerve lesion continued until Day 14 (Fig. 2C). However, the reappearance of blue stained glomeruli at Day 42 and subsequently at Day 70, indicating that the regenerating olfactory nerves had reestablished connections with the olfactory bulb. Compared to control IgG mice, significantly higher levels of the nerve recovery were found in MR16-1 mice at Day 42 and 70 in a dose-dependent manner.

Fig. 2D shows changes in the amount of injury-associated tissue on the NTx side. In control IgG-injected mice, the amount of injury-associated tissue (glial scar) consistently increased until Day 14 but decreased at Day 42 and 70. In low and high doses of MR16-1-injected mice, however, it was significantly less than that in the control IgG mice in MR16-1 dose dependent manner.

GFAP-positive cells increased on the NTx side until Day 14 and decreased at Day 42 and 70 in control IgG mice (Fig. 3). CD68-positive cells also increased in the olfactory bulbs at Day 5 but gradually decreased at D14 and after. With MR16-1 treatment, the number of both GFAP and CD68 cells decreased at all time points compared to those in control IgG mice.

3.2. Olfactory function tests

An olfactory function test using the observation of avoidance conditioning behavior to cycloheximide was administered to mice injected with MR16-1 (4 mg) or control IgG (4 mg) before and after the NTx procedure. For MR16-1 mice, 10 of 15 (67%) mice achieved a score of 100% on the olfactory function test after NTx, indicating that their olfactory function was restored. For the 10 mice that showed recovery of olfaction, the average time required for behavioral recovery was 55 ± 9 days, while only 2 of 10 (20%) control IgG mice recovered their olfactory function (required time: 44 and 88
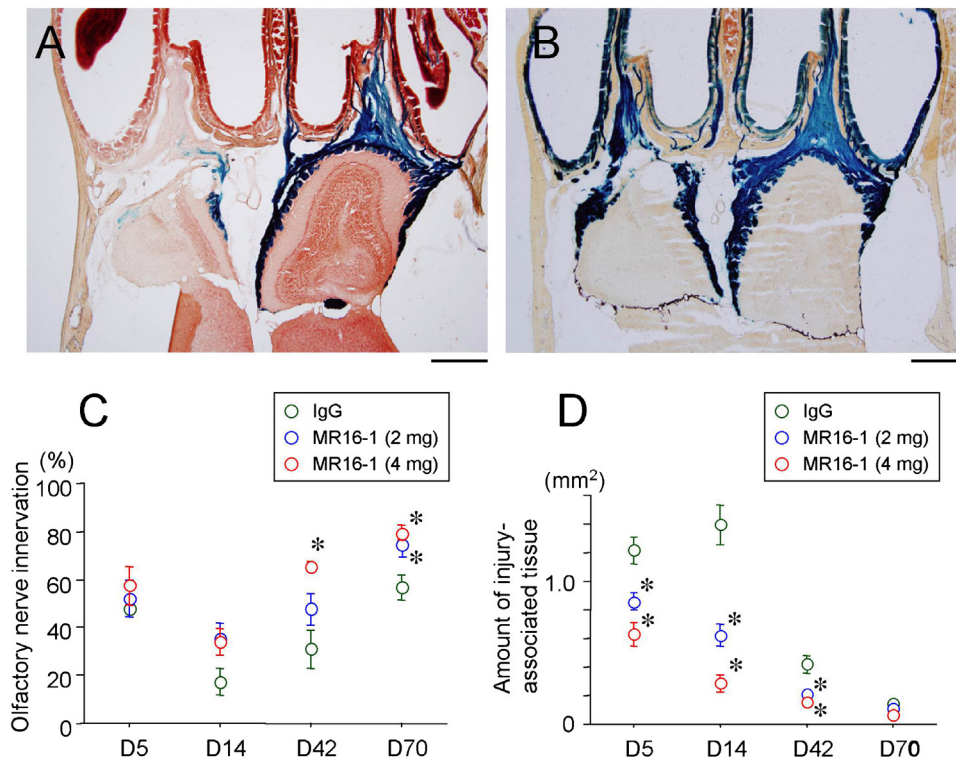
Fig. 2. Effects of MR16-1 injection on recovery from olfactory NTx injury. Histological sections illustrating control IgG-injected (2 mg, A) and MR16-1-injected (4 mg, B) animals examined at 70 days after NTx injury. Quantitative measurements showing the time course and comparison of changes in X-gal stained olfactory nerve innervation to the glomerular layer (C) and the amount of injury-associated tissue (D) for MR16-1 and control IgG-injected mice. Data plotted are means ± SE. Asterisks (*) indicate significant differences ($p < 0.0167$) compared to IgG controls. Calibration bars in A and B = 500 μm.

days). MR16-1 mice showed significantly higher rate of olfactory function recovery than control IgG mice ($p < 0.05$).

3.3. Electric field potential in olfactory bulbs evoked by olfactory mucosa stimulation

In 7 control mice, we recorded olfactory mucosa stimulation-induced field potential in the olfactory bulb before and after the bilateral olfactory NTx (Fig. 4A). In all 7 mice, a field potential was recorded before the NTx and it was completely diminished just after the NTx (Fig. 4B and C). Positive field potentials were successfully recorded from all mice that showed functional recovery in the olfactory test while little or no field potentials were observed in mice that did not recover olfaction function (Fig. 4D and E). The MR16-1 mice group showed significantly larger field potentials than the control IgG group (Fig. 4F).

4. Discussion

Olfactory receptor cells located in the olfactory epithelium are bipolar cells and their axons are connected to secondary neurons with synapse formation in the glomeruli on the olfactory bulb. Previous studies reported that olfactory bulbectomy or axotomy induced apoptosis in olfactory receptor neurons, resulting in their degeneration (Nan et al., 2001; Miwa et al., 2002). These degenerative neurons release chemical signals that initiate programmed cell death. Regeneration of olfactory receptor neurons subsequently occur by proliferation and differentiation of the globose basal cells that are progenitors of olfactory receptor neurons in the olfactory epithelium and by neurogenesis mediated by the olfactory ensheathing cells that enclose fascicles of olfactory receptor neurons. Therefore, olfactory NTx in our study is considered to give degenerative effect on the olfactory epithelium retrogradely as

well as upon the glomeruli on the olfactory bulb anterogradely, followed by regeneration of olfactory receptor neurons that were identified as blue color-stained portions in Fig. 2B (left OB, NTx side).

The present study revealed that MR16-1, anti-mouse IL-6R antibody, can suppress local infiltration of inflammatory cells and glial scar tissue formation and subsequently facilitate functional recovery of the olfactory system in a dose-dependent manner. These results are consistent with our previous report that demonstrated olfactory nerve regeneration by systemic steroid administration, suppressing local inflammation and glial scar formation in the same mouse injury model (Kobayashi and Costanzo, 2009). While our previous steroid study used only histological methods, the present study demonstrated functional olfactory recovery by electrophysiological recording methods and olfactory function testing using avoidance conditional behavior to an odorant, as well as morphological assessments of regeneration and recovery. Results of the previous and present studies are consistent though steroids and anti-IL-6R antibody are pharmacologically different, which confirms that local inflammation and gliosis can impair olfactory nerve regeneration and recovery.

IL-6R is known to be mainly expressed by hepatocytes, neutrophils, monocytes/macrophages and some lymphocytes whereas IL-6 is produced by many kinds of cells such as B cells, T cells, monocytes, fibroblasts, keratinocytes, endothelial cells, astrocytes, bone marrow stroma cells and mesangial cells (Rose-John et al., 2006; Kishimoto, 1989). IL-6 performs its signal transduction by binding with membrane bound IL-6R and subsequent combination of IL-6-IL-6R complex with membrane glycoprotein gp130 (Taga and Kishimoto, 1997). In addition to membrane bound IL-6R, there is soluble IL-6R in the body fluid, which constitutes a complex with IL-6. An interesting characteristic is that the IL-6-soluble IL-6R complex can achieve signal transduction in any cell that expresses
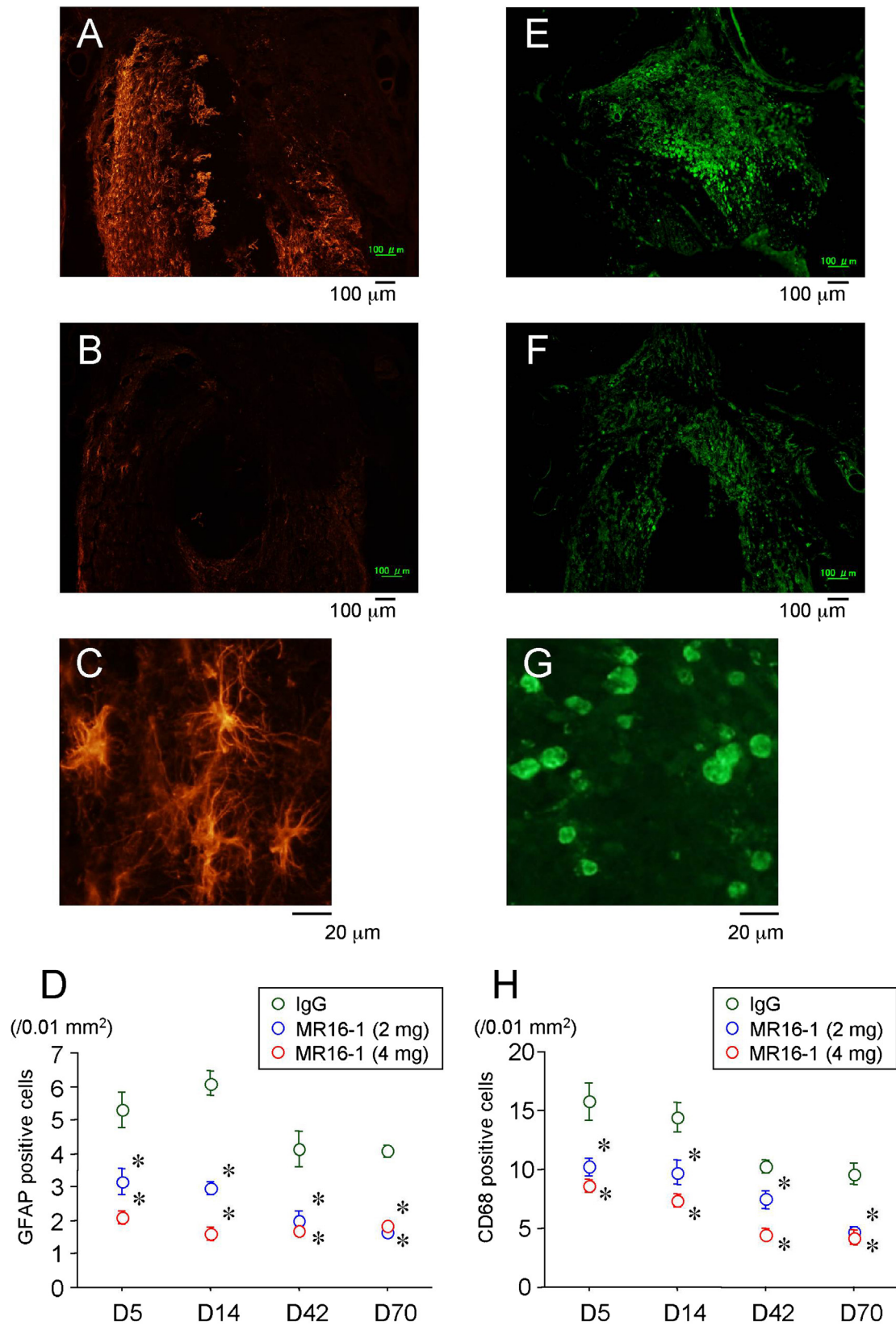
Fig. 3. Injury-related changes in GFAP-positive cells (A–D) and CD68-positive cells (E–H). Immunofluroescent staining of olfactory bulb sections from NTx-lesioned sides in control IgG-injected mice (A and E) and MR16-1-injected mice (B and F) at 5 days after NTx injury. Increases in GFAP-positive cells (golden color) and CD68-positive cells (green color) are suppressed in MR16-1 treated mice (B, F) compared to control IgG mice (A, E). Higher magnification images of GFAP-positive cells (C) and CD68-positive cells (G) were used for cell counting. Quantitative measurements showing the time course and a comparison of changes in GFAP-positive cells (D) and CD68-positive cells (H) for MR16-1 and control IgG mice. Data plotted are means ± SE. Asterisks (*) indicate significant differences ($p < 0.0167$) compared to IgG controls.
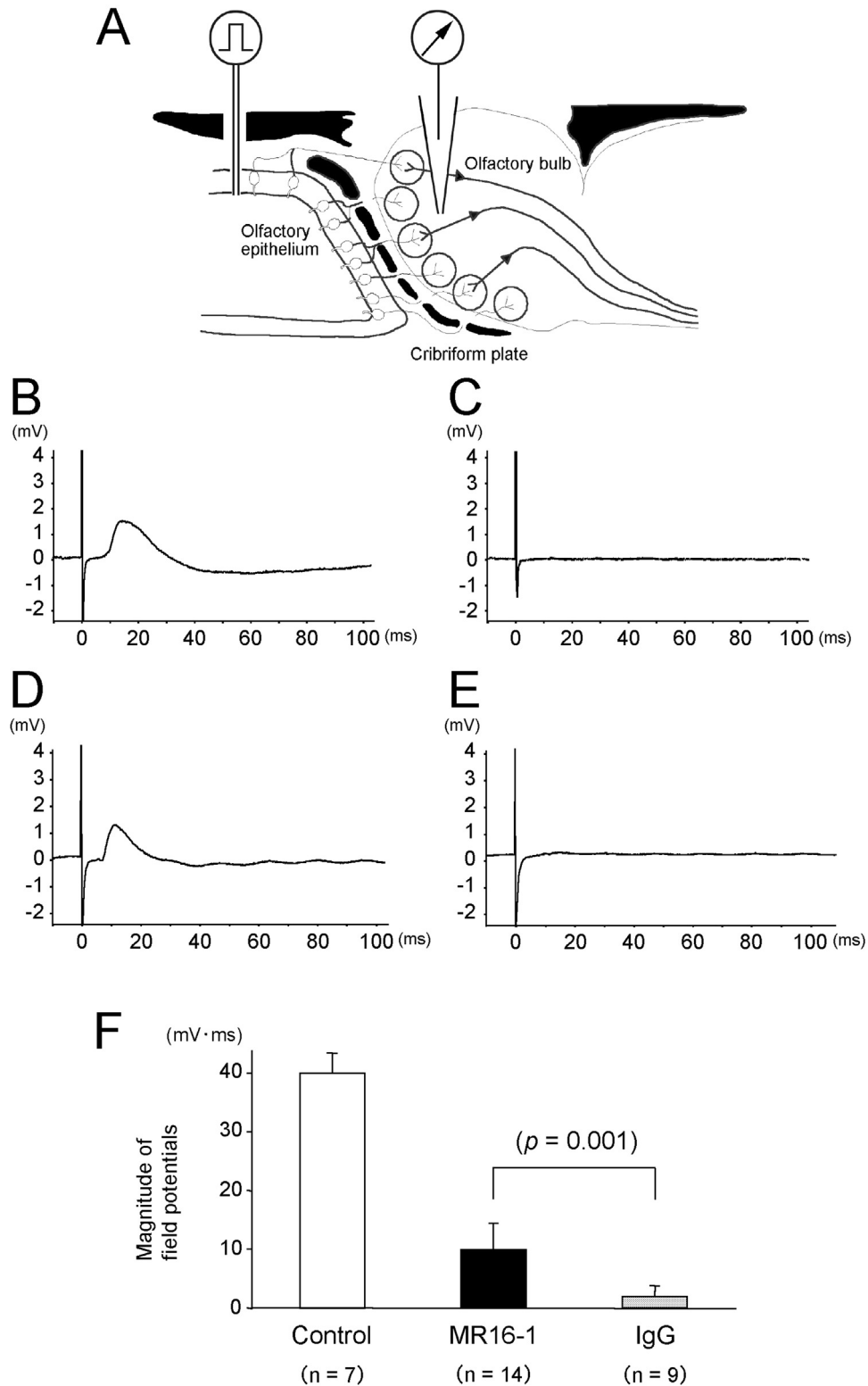
Fig. 4. Electric field potential in olfactory bulbs evoked by olfactory mucosa stimulation. (A) A schema of field potential recording. Control field potential recording before (B) and just after NTx (C). (D) Evoked field potential of a MR16-1 mouse that showed olfactory function recovery in the olfactory functional test at 40 days after the NTx injury. (E) No evoked field potential of a control IgG mouse that did not show any olfactory behavioral function recovery in the olfactory test at 100 days after the NTx injury. (F) Comparison of time integral magnitude of field potential between MR16-1 and control IgG mice, referring to that of control mice without the NTx.

gp130 even if it does not express membrane bound IL-6R. This mechanism is called "trans-signaling" and enables IL-6 to affect many kinds of cells since gp130 is expressed by almost cells. In addition, anti-IL-6R antibody can dissociate IL-6 and soluble IL-6R from these preformed complexes and it can inhibit IL-6R directly (Mihara et al., 2005). Our results suggested that IL-6R, the target site of MR16-1, exists in the injured olfactory system and MR16-1 can combine with IL-6R to block inflammatory signals.

In the present study, anti-IL-6 antibody suppressed proliferation of GFAP-positive cells in a dose-dependent manner. It is known that most of GFAP-positive cells are reactive astrocytes (Silver and Miller, 2004). When the central nervous system is injured, stem cells derived from ependymal cells proliferate and migrate to the lesion site and differentiate exclusively into astrocytes, which are eventually associated with glial scar formation (Johansson et al., 1999). Glial scar tissue is considered a physical barrier and prevents axonal regeneration by producing axonal growth inhibitors such as chondroitin sulfate proteoglycans (David and Lacroix, 2003). In addition, a previous study demonstrated that failure of regeneration after spinal cord transection is due to the prolonged disappearance of reactive astrocytes in the lesioned area (Iseda et al., 2004). This is supported by other reports that central nervous system transection does not form glial scars in the lesion when marked regeneration occurs (Varga et al., 1996; Inoue et al., 1998; Kikukawa et al., 1998). These results are consistent with our findings that animals showing less reactive astrocytes and injury-associated tissue after NTx had a better recovery of the olfactory nerves.

CD68 is expressed on macrophages as well as activated microglia, both of which appear in damaged tissue and play a role in phagocytosis. Macrophages/microglia are reported to be the major inflammatory cells in the injured central nervous system (Hurst et al., 2001; Van Wagoner and Benveniste, 1999). Therefore, we regarded CD68 a good indicator for monitoring the degree of inflammatory processes. IL-6 is known to promote the activation and infiltration of macrophages/microglia and overexpression of IL-6 makes the inflammation to worsen the tissue injury (Klusman and Schwab, 1997; Lacroix et al., 2002). Our results demonstrated that administration of anti-IL-6R antibody decreases CD68-positive cells in injured olfactory bulbs, indicating that local inflammation can be exactly suppressed by blockade of IL-6R as well as steroid administration as previously reported (Kobayashi and Costanzo, 2009).

Macrophages and microglia appearing in damaged tissue are derived from resident microglia, native macrophages of the central nervous system and hematogenous macrophages that derive from monocytes which infiltrate from the peripheral blood to the central nervous system only following damage (Popovich and Hickey, 2001). While they are considered to be functionally homogeneous so that they are indistinguishable by standard immunohistochemical techniques (Shechter et al., 2009), previous studies reported that hematogenous macrophages are more cytotoxic than microglia and their excessive infiltration into a lesion area is detrimental to central nervous system repair (Popovich et al., 1999; Gris et al., 2004). A recent study reported that MR16-1 reduced infiltration of macrophages but increased the number of microglia in the injured spinal cord (Mukaino et al., 2010). The study concluded that, since the microglia had greater phagocytic ability against myelin debris after spinal cord injury than hematogenous macrophages, switching the major inflammatory cell type from hematogenous macrophages to resident microglia at the lesion site resulted in improved tissue sparing and debris clearance, which promoted neural repair after injury. In light of these reports, most of the CD68-positive cells observed in MR16-1-injected mice in our study may be activated microglia, which could contribute to better regeneration of the olfactory system in IL-6R-blocked mice.

In this study, since olfactory mucosa stimulation-induced field potential was recorded at deeper location than the glomerular layer in the olfactory bulb, evoked field potentials indicate restoration of synapse formation between the olfactory receptor neuron and secondary neuron within the glomeruli after the NTx. Effects of IL-6 on synapse formation are controversial. A previous study reported that IL-6 inhibits synaptic plasticity in the hippocampus (Tancredi et al., 2000), while other studies suggested that IL-6 contributes to synapse formation in the cerebellium and spinal cord (Wei et al., 2011; Yang et al., 2012). Though there are no reports demonstrating effects of IL-6 on synapse formation in the olfactory system, based on our results, blockade of IL-6 signal transduction may contribute to synapse formation at the glomeruli on the olfactory bulb indirectly through suppression of local inflammatory reaction, considering that inflammation and glial scar are critical factors to inhibit axonal regeneration, Our present results suggest that anti-IL-6R antibody can be a promising drug for the treatment of olfactory dysfunction after head injury to replace steroids, especially considering the clinical consideration that administration of steroids to patients with head trauma is not recommended. However, some studies reported, using IL-6 gene-knockout animals, that complete blockade of IL-6 signaling is detrimental to functional recovery of the injured central nervous system by inhibiting axonal regeneration or causing failed gliosis and tissue necrosis (Swartz et al., 2001; Cafferty et al., 2004; Okada et al., 2006). These reports indicate that IL-6 has two conflicting roles that are unfavorable and beneficial to central nervous system repair. Therefore, the practical use of anti-IL-6R antibody in humans will require proper dosing and administration and must be decided carefully. Our results showed that the dose and timing used in our study is appropriate for good recovery in the olfactory system. In many clinical cases of head injury, however, olfactory impairment is not diagnosed until weeks or months after the injury since patients and medical staff usually direct their attention to more critical life threatening injuries and often overlook any olfactory loss. Future studies are needed to determine effective timing and dose for treatment using anti-IL-6R antibody during chronic olfactory dysfunction that occurs at later stages following injury.

Acknowledgments

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