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Tumor necrosis factor-α antagonist suppresses local inflammatory reaction and facilitates olfactory nerve recovery following injury

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ABSTRACT

Objective: Olfactory dysfunction is a common finding in head trauma due to injury to the olfactory nerve. We previously reported that anti-inflammatory treatment with steroids improves recovery outcome in olfactory nerve injury models. Clinically, however, steroid administration is not recommended in the acute phase of head injury cases because of concerns regarding its side effects. Tumor necrosis factor (TNF-α) is known to play a key role in inflammatory response to injury. The present study examines if the inhibition of TNF-α can facilitate functional recovery in the olfactory system following injury.

Materials and methods: Olfactory nerve transection (NTx) was performed in olfactory marker protein (OMP-tau-lacZ) mice to establish injury models. We measured TNF-α gene expression in the olfactory bulb using semi-quantitative and real time polymerase chain reaction (PCR) assays and found that they increase within hours after NTx injury. A TNF-α antagonist (etanercept) was intraperitoneally injected immediately after the NTx and histological assessment of recovery within the olfactory bulb was performed at 5–70 days. X-gal staining labeled OMP in the degenerating and regenerating olfactory nerve fibers, and immunohistochemical staining detected the presence of reactive astrocytes and macrophages/microglia.

Results: Etanercept-injected mice 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 recordings showed improved functional recovery in etanercept-injected animals.

Conclusion: These findings suggest that inhibition of TNF-α could provide a new therapeutic strategy for the treatment of olfactory dysfunction following head injuries.

1. Introduction

Olfactory dysfunction is a common finding in head trauma due to injury to the olfactory nerve. The incidence of olfactory dysfunction due to head trauma is reported to be 5–26% [1,2]. Olfactory dysfunction lowers our quality of life and can be life threatening because of the inability to detect hazardous events such as fire, gas leak, and spoiled food intake [3]. Causes of olfactory dysfunction in head trauma are overextension, distortion and tearing of the olfactory nerves and contusions of the olfactory bulbs and orbitofrontal regions of the brain [4]. Although the olfactory system has a remarkable capacity for neural regeneration and recovery following injury, prognosis of head trauma patients for recovery from olfactory dysfunction is only 10–38% [2,5] while that in patients with chronic rhinosinusitis and allergic rhinitis is reported to be

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better, with recovery rates of up to 68–86% [6,7]. So, development of therapeutic management for improving olfactory dysfunction is an important clinical objective.

We previously demonstrated using an olfactory nerve injury model in mice that anti-inflammatory treatment with steroids or anti-interleukin-6 (IL-6) antibody during the acute phase of injury is effective in suppressing the inflammatory reaction and local glial scar formation and improves recovery outcomes after olfactory nerve transection (NTx) [8,9]. In clinical practice, however, these drugs 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 [10,11]. Although there are fewer concerns regarding anti-IL-6 antibody use, its administration may sometimes induce severe infection [12].

Tumor necrosis factor (TNF)-α is a key cytokine that plays a central part in inflammation, immunity and apoptosis, and is released in response to stress and injury [13]. A TNF-α antagonist, etanercept, has been reported to be an effective inhibitor of the inflammatory reaction by preventing TNF-α from combining with the TNF-α receptor expressed on surface of cells and has been used instead of steroids for the treatment of refractory inflammatory diseases such as rheumatoid arthritis [14]. In the central nervous system, an increase in the levels of TNF-α occurs shortly after injury and it is linked to many factors like cellular dysregulation, enhanced vascular permeability and impaired glutamate metabolism, and sometimes to excessive inflammatory reactions [15]. A recent study reported that administration of etanercept suppresses the severity of the response to trauma and facilitates functional recovery in experimentally induced spinal cord injury in mice [16].

The present study was designed to investigate if therapeutic intervention using TNF-α antagonist is effective in improving recovery outcomes in the olfactory system following injury in mice. In this study, we first demonstrated an increase in levels of TNF-α in the injured olfactory system using a polymerase chain reaction (PCR) assay, which would be a target of the TNF-α antagonist. Subsequently, we used histological techniques to examine the efficacy of the TNF-α antagonist on recovery outcome by measuring the degree of degeneration and regeneration of olfactory nerve fibers and the amount of injury-associated tissue (glial scar), reactive astrocytes and macrophages/microglia. We also administered an olfactory function test using avoidance conditioning behavior to odorants as well as electrophysiological recording of field potential responses to electrical stimulation of the olfactory mucosa to determine if morphological recovery parallels functional recovery in the olfactory system following therapeutic intervention.

2. Material and methods

2.1. Experimental animals

This study was performed using transgenic mice (OMP-tau-lacZ mice) obtained from the Jackson Laboratory (Bar Harbor, ME, USA), whose strain is derived from C57BL/6 mice. In this strain the gene sequence encoding the olfactory marker protein (OMP) has been replaced with a tau-lacZ reporter gene [17]. The OMP is expressed in all mature olfactory neurons [18] and the replacement with tau-lacZ reporter gene enables the visualization of olfactory nerve fibers and their projections to olfactory bulb glomeruli. The advantage of using these mice is that a histological assessment of degenerating and regenerating olfactory nerve fibers can be performed using a standard method for staining and light microscopy.

2.2. Surgical procedure

Both male and female adult mice were used in this study and randomly assigned to experimental groups. Mice were anesthetized with sodium pentobarbital (80 mg/kg, *ip*). Under sufficient anesthesia, a frontal craniotomy was performed to expose the olfactory bulbs. An olfactory nerve transection procedure (NTx) was performed between the olfactory bulb and cribriform plate using a curved rigid stainless steel blade to generate a severe olfactory nerve injury model [8]. For histological assessments, the NTx procedure was performed only 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. 1). For other assessments (PCR, olfactory function testing, evoked potentials), a bilateral NTx was performed, cutting the olfactory nerves to the right and left olfactory bulbs, resulting in a complete loss of smell (anosmia). After the NTx procedure 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 of Mie University.

2.3. PCR

For the PCR assays, mice ($n = 21$) were divided into groups according to the time of sacrifice after the bilateral NTX surgery (1, 3, 6, 12, 24, 48 h). An additional control group (0 h) was included that did not have any surgery. The olfactory bulbs were removed immediately after sacrifice and the anteroventral part sectioned off and stored at 80 °C for semi-quantitative and real-time PCR TNF-α assays.

The olfactory bulb tissue samples were placed into Sepasol Super G and homogenized. Total RNA was extracted and purified by DNase treatment according to the manufacturer's protocols. The reverse transcription reaction was performed using the RevertraAce (Toyobo, Osaka, Japan) using 1 microgram of total RNA. Variation in the reverse transcription reaction was limited by transcribing all samples simultaneously. PCR reactions were optimized to 94 °C for 2 min, 45 amplification cycles for TNF-α and 26 cycles for GAPDH at 94 °C for 10 s, 60 °C for 20 s, 72 °C for 40 s, and a final extension of 2 min at 72 °C. Amplified products were resolved on 1.5% agarose gel and visualized by ethidium bromide staining using ITLS software.

Real time PCR reactions were performed using the AB step One system with SYBR Green PCR Mastermix (Applied

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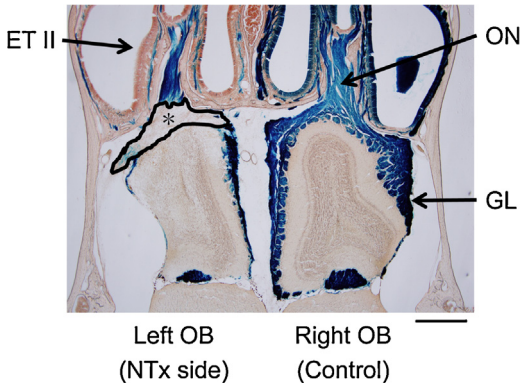

Fig. 1. An experimental mouse model of severe olfactory bulb deafferentation injury. A horizontal section through the nasal cavities and olfactory bulbs illustrating differences observed between the lesioned (left) and control (right) sides at 5 days after a nerve transection (NTx) injury. The area of injury-associated tissue (glial scar) formed between the cribriform plate and olfactory bulb (enclosed by a black line, *) was measured and quantified using Image J software (ver. 1.36, NIH, USA). The olfactory nerves and their projections to glomeruli are labeled using an X-gal staining method (blue color). GL, glomerular layer; OB, olfactory bulb; ON, olfactory nerve; ET II, endoturbinate II. Calibration bar = 500 μm.

Biosystems). The relative quantification was determined using the $2^{-\Delta Ct}$ method; GAPDH was used as an internal control to normalize the data.

Primer Sequences
TNF-α: Primer Bank ID 133892368c3
Forward: CCTGT AGCCC ACGTC GTAG
Reverse: GGGAG TAGAC AAGGT ACAAC CC
Amplicon size: 148 bp
GAPDH: Primer Bank ID: 126012538c3
Forward: TGGCC TTCCG TGTTC CTAC
Reverse: GAGTT GCTGT TGAAG TCGCA
Amplicon size: 178 bp

2.4. TNF-α antagonist injection

To investigate the associations among TNF-α, the inflammatory reaction and nerve regeneration after injury, the TNF-α antagonist, etanercept (Pfizer, Tokyo, Japan) was injected intraperitoneally just after the NTx. To determine if there is a dose-dependent effect of the drug, low (0.1 mg in 0.2 ml saline) and high (0.2 mg in 0.2 ml saline) doses of etanercept were used making them comparable to doses used in previous studies [15,16]. We also injected saline (0.2 ml) intraperitoneally in a group of control animals. We collected data from 6 mice for each of the 3 treatment groups and each of the 4 recovery time points (Day 5, 14, 42 and 70) for a total of 72 mice (6 mice × 3 doses × 4 recovery points).

2.5. Tissue preparation

For histological assays, mice were anesthetized on the assigned post recovery day with sodium pentobarbital (80 g/kg) and fixed by intracardiac perfusion using 4% paraformaldehyde in phosphate puffer after a saline rinse. The nasal cavity and anterior portion of the skull were removed *en bloc* and postfixed by immersion in 4% paraformaldehyde for 45 min and then placed in 0.5 M EDTA (ethylenediaminetetraacetic acid) for decalcification for 14 days. The tissue was cryoprotected with 30% sucrose for 2 days, then immersed in embedding compound, quickly frozen in a −80 °C freezer and sectioned on a cryostat. Serial horizontal sections along dorsum nasi were cut at 30 μm and mounted on glass slides.

2.6. 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 overnight in the dark by exposure 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.

2.7. Measurement of injury-associated tissue and nerve recovery

After confirming the appearance of the blue X-gal reaction, tissue sections were counterstained with 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. 1). 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. 1) 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 (Fig. 1). 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 mean values for injury-associated tissue. The 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

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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:

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

2.8. Immunohistochemical assessment

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 central nervous system injury, hypertrophic reactive astrocytes increase their expression of GFAP [19]. CD68 staining was used to measure injury-induced inflammatory changes at different time points after NTx injury. CD68 is a lysosomal membrane-associated 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 NTx mice at each of the four recovery time points.

2.9. Olfactory function test

To determine if olfactory function recovered after the NTx, a smell detection test using avoidance conditioning behavior to cycloheximide was administered to mice before and after the NTx as reported previously [16]. 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 NTx surgery, mice were conditioned in two or more training sessions, each consisting of 10 trials. In each trial, the mouse was presented with bottles of 0.01% cycloheximide solution and distilled water one positioned on the left the other on the right side of a test cage. When the mouse licked the delivery tube of either bottle, the bottles were 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%) on 2 two consecutive test sessions. After NTx surgery, the test was administered every 7 days until the mouse regained its olfactory function (scored 10 out of 10 correct responses), or exceeded a 105-day cut off period. Mice that scored 100% at one of the recovery test days were considered to have fully recovered their olfactory function.

2.10. Evoked 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 measured in olfactory bulb of mice that completed the cycloheximide olfactory function test. For this group of mice, the NTx procedure was bilaterally resulting in a complete loss of olfactory function. On Day 105, the animal was anesthetized and the nasal bone 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) that delivered a constant current stimulus of 0.5 mA, 0.3 ms, at a rate of 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 microelectrodes used in this experiment were glass capillary pipettes filled with 0.5 M KCl with a tip resistance of 3–6 MΩ. To improve the signal-to-noise ratio of recordings, the olfactory mucosa stimulation-induced responses were averaged for 32 stimulus trials. The magnitude of field potential was quantified by measuring the time integral of the evoked field potential.

2.11. Statistical analysis

All numerical data obtained are 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 Fisher's PLSD method for a gene expression study and by the Bonferroni method for analyses of a histological study. 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.

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3. Results

3.1. TNF-α expression

A semi quantitative PCR shows that there are significant increases in TNF-α gene expression 1 h after the NTx or later (Fig. 2A and B). Real time PCR confirmed these results as shown in Fig. 2C. While statistically significant increases in TNF-α gene expression was observed only 3 and 6 h after the NTx in real time PCR due to small number samples and multi-time point analyses of variance, these results of both PCR showed that TNF-α, the target of etanercept, increased at damaged olfactory tissues after the NTx.

3.2. Effect of TNF-α antagonist injection

To determine if TNF-α antagonist treatment can facilitate recovery of the olfactory nerves after NTx injury, etanercept was injected intraperitoneally in the severe injury model. Fig. 3A shows results of the vehicle control (saline) compared to effects of etanercept treatment (Fig. 3B). A decrease in the percentage of X-gal (blue) staining on the NTx side at Days 5 and 14 reflect the degeneration of olfactory nerves (Fig. 3C). However, the subsequent increase in blue staining in the nerve and glomerular layers at Day 42 and Day 70 indicate that the regenerating olfactory nerves had reestablished connections with the olfactory bulb. Compared to the saline controls, a significantly higher level of the nerve recovery was found in the etanercept mice at Day 42 and 70, and these increases were dose-dependent.

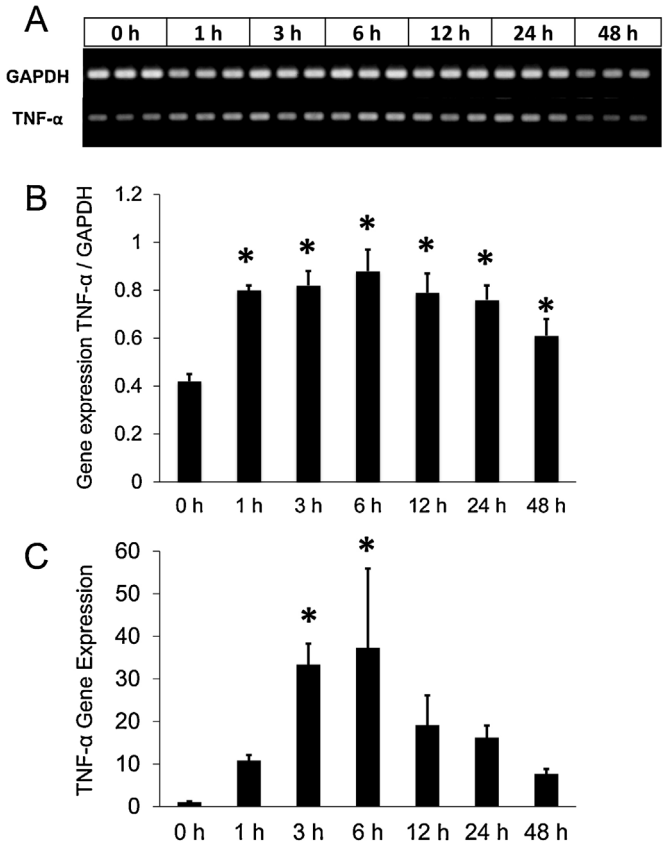

Fig. 2. TNF-α expression. (A) PCR data for GAPDH and TNF-α present in the olfactory bulb 0–48 h after NTx injury. (B) Results of semi-quantitative PCR measurements. Ratio of TNF-α genes at 1–48 h time points after NTx injury are significantly increased compared to the 0 h time point. $^{*}p < 0.05$. (C) Results of real-time PCR confirming results of the semi-quantitative PCR with a maximum peak level at 6 h after the NTx. $^{*}p < 0.05$ compared to data at 0 h time point.

Fig. 3D shows changes in the amount of injury-associated tissue (glial scar) present on the NTx side. The amounts increased at Day 5 and gradually decreased at Day 14 and after. The tissue amount in low and high doses of etanercept-injected mice was significantly less than that in control (saline) mice.

Both GFAP-positive cells and CD68-positive cells increased on the NTx side in the olfactory bulbs at Day 5 and gradually decreased at Day 14 and later time recovery times (Fig. 4). With etanercept treatment, the number of both GFAP and CD68 cells decreased compared to those in control saline mice in a dose-dependent manner.

3.3. Olfactory function tests

An olfactory function test using avoidance conditioning behavior to cycloheximide was administered to mice injected with etanercept (0.2 mg in 0.2 ml saline) the saline (0.2 ml) control before and after the NTx. For the etanercept group, 13 of 14 (93%) mice achieved a score of 100% on the olfactory function test after NTx, indicating that their olfactory function had recovered (Table 1). The average time required for behavioral recovery in the 13 mice was 64 ± 5 days. For the control saline group, however, only 3 of 11 (27%) mice recovered their olfactory function (91, 70, 84 days, 82 ± 6 days). Etanercept-injected mice showed a significantly higher rate of olfactory function recovery than control saline-injected mice ($p < 0.05$).

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

Positive field potentials induced by electrical stimulation of olfactory mucosa were successfully recorded from the 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. 5). The etanercept group showed significantly larger field potentials than the saline control group.

4. Discussion

The present study shows that etanercept, a TNF-α antagonist, can suppress local infiltration of inflammatory cells and glial scar tissue formation and subsequently facilitate morphological and functional recovery of the olfactory system. These results are consistent with our previous reports that demonstrated olfactory nerve regeneration by systemic administration of steroid and anti-IL-6 antibody, both of which suppress local inflammation and glial scar formation in the mouse injury model [8,9]. Results of the previous and the present study are consistent although the TNF-α antagonist is pharmacologically different from steroids and anti-IL-6R antibody, which means that local inflammation and gliosis

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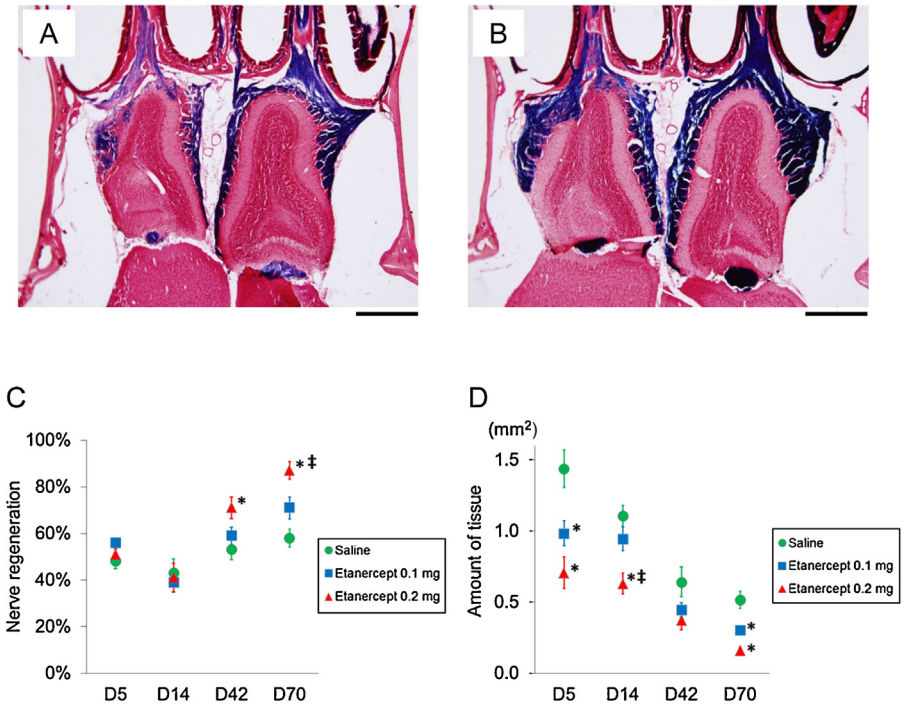

Fig. 3. Effects of TNF-α antagonist injection on recovery. Effects of etanercept on recovery from olfactory NTx injury. Histological sections illustrating control saline (0.2 ml, A) and etanercept (0.2 mg, B)-injected mice at 70 days after NTx injury. Quantitative measurements showing the time course and comparison of changes in X-gal stained olfactory nerve innervation to the glomerular layer on the olfactory bulb (C) and the amount of injury-associated tissue (D) for etanercept (low and high doses) and control saline animals. Asterisks (*) indicate significant differences ($p < 0.0167$) compared to the control saline group. Double dagger (‡) indicate significant differences ($p < 0.0167$) between the low and high dose etanercept groups.

can suppress olfactory nerve regeneration and recovery and that nonspecific anti-inflammatory treatment can be useful for olfactory system recovery after nerve injury.

In our study, we measured degree of reinnervation of olfactory nerve axons to glomeruli on the olfactory bulb as an indicator of olfactory system restoration. A recent study showed that using mouse models of olfactory loss by chronic rhinosinusitis, regeneration of olfactory epithelium is also facilitated by etanercept administration [20]. While we did not examine changes in olfactory epithelium, both of the previous and present reports proved that anti-inflammatory intervention by etanercept is useful for olfactory system restoration.

Pathological progress of traumatic injury in the central nervous system as observed in the spinal cord consists of two chronological phases, the primary injury and the secondary injury [21]. Within 24 h after the primary injury caused by direct mechanical damage to a neuronal tissue, proinflammatory cytokines are produced and released at the lesion site in the second phase. These cytokines induce expansion of tissue damage by increase in vascular permeability, massive recruitment of inflammatory cells such as hematogenous neutrophils and macrophages/microglia, which produce proteolytic enzymes and reactive oxygen species causing degeneration of myelin and apoptosis of neurons. These reactions lead to cell death, Wallerian degeneration and scar formation in the nervous system, resulting in neurological deficits. TNF-α is one of the trigger cytokines that initiate this reaction cascade in the secondary phase of the trauma process. It is not usually detectable in healthy individuals but is produced mainly by macrophages/microglia and monocytes in serum and tissue in response to occurrence of inflammation and infection [22,23]. Our results showed that TNF-α at the lesion site significantly increased after NTx injury. Therefore, targeting TNF-α is considered to be a reasonable strategy for suppression of the inflammatory reaction and prevention of neural damage after traumatic injury.

TNF-α can bind to its specific receptors, TNFR1 and TNFR2, to achieve signal transduction. All nucleated cells, except erythrocytes, express TNF receptors while their distribution is variable among different cell types. TNFR1 is usually expressed on most cells whereas expression of TNFR2 is induced in astrocytes and hematopoietic cells when cytokines such as TNF-α, interleukin-1 and interferon-γ stimulate [24,25]. Etanercept, TNF-α antagonist, is a genetically engineered fusion protein with two chains that are identical to recombinant TNFR2 [26]. Etanercept is composed of two TNFR2 and Fc portion of IgG so that it can bind to two TNF-α molecules and block their interaction with the TNF receptors expressed on most cells, resulting in inactivation of TNF-α signal transduction. Although blockade of excessive TNF-α function using etanercept is beneficial to suppression of the inflammatory reaction in traumatic injuries and functional restoration of organs in the spinal cord, some studies reported that low levels of TNF-α possess beneficial function as maintenance of homeostasis by regulating circadian rhythm, remodeling and replacement of injured and senescent tissue by stimulating fibroblast growth, immune response to bacterial and viral invasion and necrosis of specific tumors [27,28]. These reports suggested that TNF-α has two conflicting roles that are unfavorable and beneficial to traumatic system restoration.

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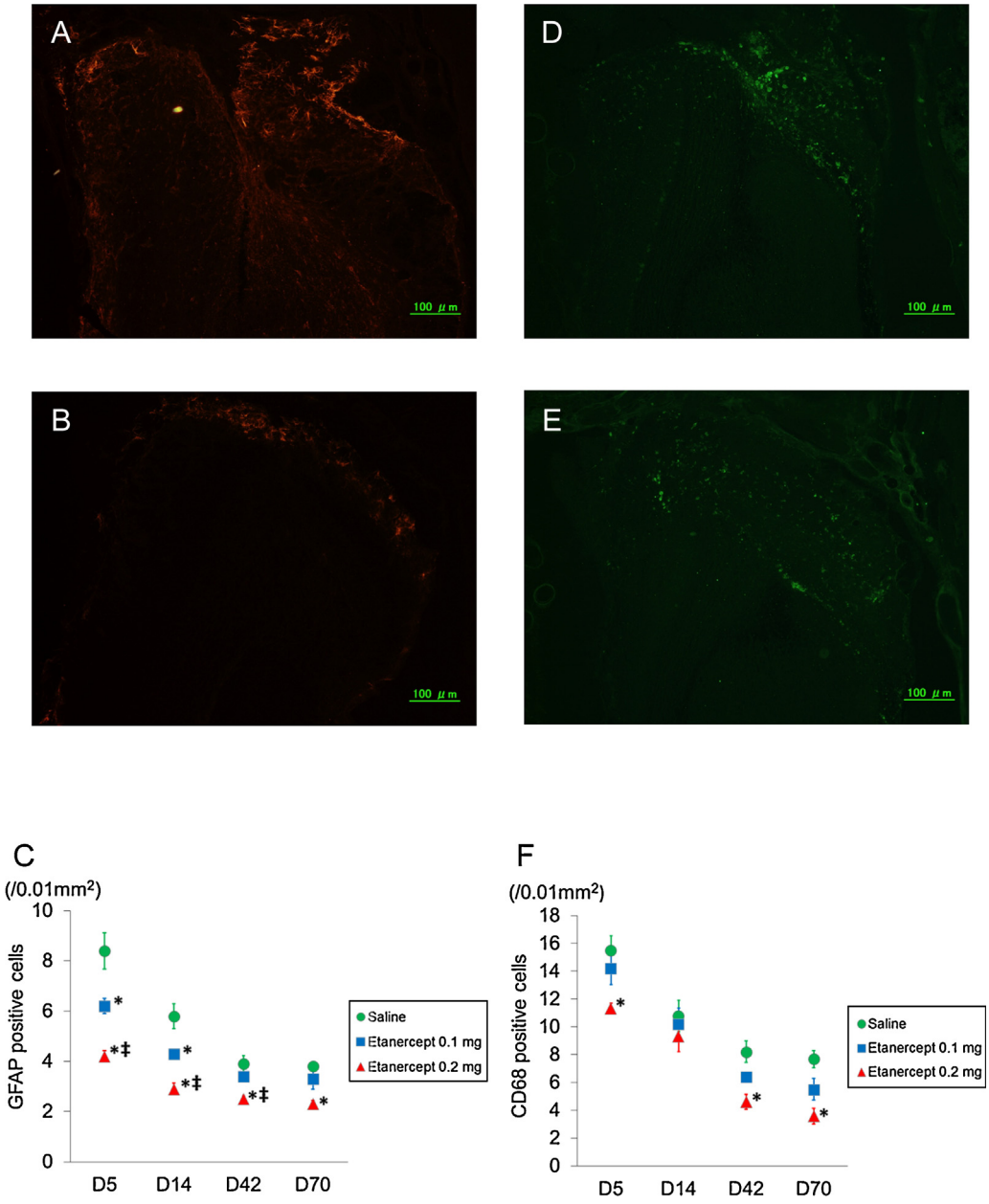

Fig. 4. Effects of TNF-α antagonist injection on glial and inflammatory cells. Injury-related changes in GFAP-positive cells (A–C) and CD68-positive cells (D–F). Immunofluorescent staining of olfactory bulb sections from NTx-lesioned sides in control saline-injected mice (A and D) and etanercept-injected mice (B and E) at 70 days after NTx injury. Increases in GFAP-positive cells (red color) and CD68-positive cells (green color) are suppressed in etanercept treated mice (B and E) compared to control saline mice (A and D). Quantitative measurements showing the time course and a comparison of changes in GFAP-positive cells (C) and CD68-positive cells (F) for etanercept and control saline mice. Data plotted are means ± SE. Asterisks (*) indicate significant differences ($p < 0.0167$) compared to saline controls. Double daggers (‡) indicate significant differences ($p < 0.0167$) between the low and high dose etanercept groups.

Table 1
Results of the olfactory function test.

	Saline	Etanercept	
Recovering/total mice (n/n)	3/11	13/14	
% of recovering mice	27%	93%	$p < 0.05$
Needed days for recovery	82 ± 6	64 ± 5	N.S.

Therefore, the optimal dose of etanercept should be decided carefully for damaged tissue repair. The dose used in this study may be appropriate for damaged in the olfactory system, considering the results showing good recovery of olfactory system.

Our result demonstrated that TNF-α antagonist suppressed proliferation of reactive astrocytes that are identified as GFAP-positive cells in the olfactory lesion area. It is known that reactive astrocytes are associated with glial scar formation [29]

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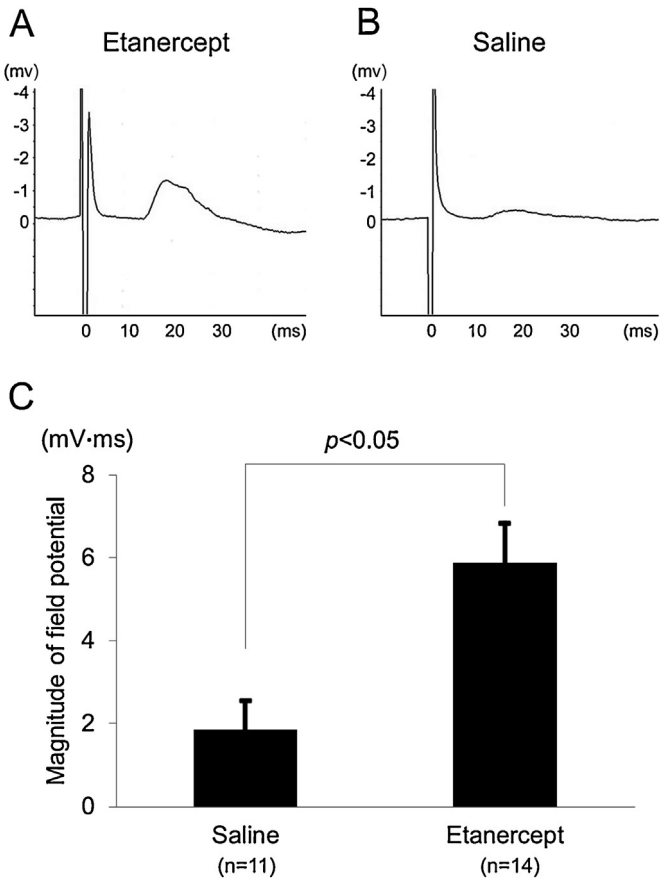

Fig. 5. Electric field potential in olfactory bulbs evoked by olfactory mucosa stimulation. Evoked field potential of an etanercept mouse that showed olfactory function recovery (A) and almost no evoked field potential of control saline mouse that did not show any olfactory behavioral function recovery (B), in the olfactory functional test at 105 days after the NTx injury. (C) Comparison of the time integral magnitude of field potentials, between the etanercept (0.2 mg) and control saline groups.

and their prolonged appearance in the lesion area leads to less regeneration of the central nervous system through production of axonal growth inhibitors such as chondroitin sulfate proteoglycans [30]. In addition, while astrocytes support neuronal growth, cytokines like TNF-α secreted in injured central nervous system can alter astrocytes physically and chemically [31]. TNF-α stimulates the NF kappa B pathway in astrocytes, leading to reduced glutamate uptake from the synaptic cleft which will compromise the neuronal survival due to prolonged excitation of these cells by excessive glutamate in the synaptic cleft [32]. Furthermore, previous studies reported that TNF-α stimulation of astrocytes results in upregulation of IL-6 leading to exacerbation of the inflammatory response [33] and that ablation of reactive astrocytes promoted neurite outgrowth after injury in a transgenic mice [34]. These results coincide with our findings that the mice showing less reactive astrocytes and injury-associated tissue after NTx injury acquire a better recovery of the olfactory nerves.

In our studies, CD68 was used as an indicator of the degree of inflammatory reaction since it is expressed on macrophages as well as activated microglia [8,9], both of which accumulate in damaged tissue, perform phagocytosis and are reported to be the major inflammatory cells in injured central nervous system [35]. While macrophages produce TNF-α in response to inflammation or injury, TNF-α induces migration of hematogenous macrophage into damaged tissue by increasing vascular permeability [21]. Infiltration of peripheral macrophages leads to lesion enlargement and progressive tissue destruction since they release proinflammatory cytokines [36]. In addition, a previous study reported that depletion of the macrophages contributes to sparing of axons of nerves and improvement of neuronal activity [37]. These are consistent with present findings that local inflammation can be exactly suppressed by inhibition of TNF-α activity using etanercept, which results in a better restoration of damaged olfactory system by the NTx.

In this study, electrophysiological examination was performed to confirm functional recovery of the olfactory system in addition to olfactory function tests using avoidance conditioning behavior. A previous study demonstrated using spinal cord injury model in rabbits that results of electrophysiological recording of somatosensory evoked potential were significantly better in etanercept-administered animals than that in control group [13]. In addition, those results were compatible with scores of functional assessment of lower limb movement. Our results in electrophysiological examination are also parallel with results in olfactory function test, which confirm that etanercept-injected mice obtained functional recovery, including restoration of synapse formation between the olfactory receptor neuron and secondary neuron within the glomeruli after the NTx.

Our results suggest that etanercept can be a promising drug for the treatment of olfactory dysfunction in the acute phase of head trauma instead of steroids, especially considering the clinical indication that steroids are not recommended for patients with head injury. In addition, our results show that the timing used in this study is appropriate for good recovery of olfactory system. In many clinical head injury cases, however, olfactory dysfunction 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 impairment. One report suggested that treatment with etanercept that was delayed for 14 days after spinal cord injury was not effective in mice [15]. Therefore, future studies are needed to determine effective timing for treatment using etanercept during chronic olfactory dysfunction that occurs at later stages following head trauma, as well as optimal dose and combination with other drugs having synergistic effects.

5. Conclusions

The present study revealed that inactivation of TNF-α using its antagonist, etanercept, in an acute phase of olfactory nerve injury can contribute to ameliorate functional restoration of the olfactory system by suppressing local infiltration of inflammatory cells and glial scar tissue formation and may provide a new therapeutic strategy for the treatment of olfactory dysfunction following head injuries.

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Conflict of interest

The authors declare that they have no conflict of interest.

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