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Research Paper
Distraction Osteogenesis

Co-expression of nerve growth factor and p75NGFR in the inferior alveolar nerve after mandibular distraction osteogenesis

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Abstract. During mandibular distraction osteogenesis (DO), the inferior alveolar nerve (IAN) is damaged during distractor activation, but spontaneously recovers during consolidation. Although many neurotrophic factors are known to play critical roles, there have been few studies on the mechanism of peripheral nerve recovery after DO. The aim of this study was to observe the expression pattern of p75NGFR (low-affinity receptor of NGF) and to detect autocrine growth activity in IANs following mandibular DO. Unilateral mandibular distractions (0.5 mm each, twice per day for 10 days) were conducted on eight mongrel dogs. Two each were killed at 7, 14, 28 and 56 days after completing distraction. The distracted IAN and contralateral control nerve were harvested. Immunohistochemical staining was performed to determine p75NGFR expression, and double immunofluorescent staining to detect NGF and p75NGFR co-expression. Levels of p75NGFR expression were found to be significantly elevated at 7 and 14 days in Schwann cells located in the outer layer of axon, but were almost undetectable at 28 and 56 days. In double immunofluorescent images, the co-expression of NGF and p75NGFR was also detected at 7 and 14 days. p75NGFR plays an important role in remyelination due to its abundant expression in Schwann cells of damaged nerves, and NGF is an autocrine growth factor present in distracted IANs during the early consolidation period after mandibular DO.

Keywords: mandibular distraction osteogenesis; inferior alveolar nerve; p75NGFR; autocrine growth activity.

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Mandibular distraction osteogenesis (DO) is broadly used in the reconstruction of mandibular defects. Sensory disturbances have been reported in 25–57% of patients after mandibular DO, because the inferior alveolar nerve (IAN) bundle is positioned

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within the mandibular canal[10]. The IAN may be easily stressed within the canal during mandibular DO, but it is well known that no significant nerve damage occurs when distraction is performed gently at 1 mm/day[1,9,11]. Several investigators[9–11] have reported that Wallerian degeneration occurs in IANs after gradual mandibular distraction. The recovery process has also been shown to occur simultaneously.

Although the precise mechanism of this peripheral nerve regeneration is not understood, it is believed that Schwann cells and various neurotrophic factors play important roles in the differentiation, regeneration and maintenance of peripheral nerve[4,10]. In a previous animal study[3], nerve growth factor (NGF) and brain-derived neurotrophic factor were found to be widely expressed in the IAN after mandibular DO. The present authors also observed[19] widespread NGF and vascular endothelial growth factor expression in IAN during the early consolidation period after mandibular DO.

NGF is the first and best-characterized nerve-derived factor. It is a 26-kDa nonglycosylated, homodimeric polypeptide and was originally identified as a neurotrophic factor and purified from male mouse salivary glands[13]. NGF acts at two distinct receptors, a 140-kDa low/high-affinity receptor (tyrosine kinase receptor A, trkA) and a 75-kDa low-affinity receptor (low-affinity nerve growth factor receptor, p75NGFR), and it dominantly affects the sensory and sympathetic nervous systems[4]. During peripheral nerve regeneration or remyelination, Schwann cells induce various neurotrophic factors and their receptors. The interactions between neurotrophic factors and their receptors during IAN recovery after mandibular DO are not understood.

Neurotrophic factors and their receptors are co-expressed in several uncommon situations, such as in the nervous system during the embryonic period[6], in some regions of the cerebral cortex[12,20] and in axotomized peripheral nerves[23]. In these situations, neurotrophic factors may have autocrine growth activity. The aims of this study were to observe the expression pattern of low-affinity NGF receptor (p75NGFR) in the IAN after mandibular DO, and to detect the autocrine growth activity in the distracted IAN by demonstrating the co-expression of NGF and p75NGFR using double immunofluorescent staining.

Materials and methods

Animal model and surgical protocol

The specimens and the animal model utilized, and surgical protocol adopted in the present study, were identical to those used in the previous study[19]. Briefly, eight mongrel dogs, aged between 1 and 2 years and weighing approximately 10 kg were used. All experimentation was authorized by the Animal Center for Medical Experimentation at Gyeongsang National University.

Under general anaesthesia, the right mandible was exposed after sequential submandible dissection. After buccal and lingual corticotomy, a mandibular distractor (Leibinger, Germany) was positioned on the buccal cortical bone taking care not to damage the IAN. The wound was closed in two layers with 3-0 Vicryl and Nylon. First generated cephalosporin (20 mg/kg; Cefazolin®, Yuhan Corp., Korea) was injected intramuscularly twice a day for 5 days after surgery. After a 5-day latency period, the mandible was distracted twice a day for 10 days at a rate of 1.0 mm/day.

Specimen preparation

After the administration of general anaesthesia, 2 animals each were killed by KCl injections at 7, 14, 28 and 56 days after completing distraction. Right distracted mandibles were then immediately harvested *en bloc*, and the elongated segments of IANs in the distracted callus were carefully dissected and separated (distraction group). The left undistracted mandibles were also block resected, using an identical procedure, and normal IANs were harvested (control group).

Immunohistochemical staining

Tissue specimens were immersed in 10% neutral buffered formalin for 24 h, and then embedded into paraffin blocks for immunohistochemical studies. In brief, paraffin blocks were cut into 4-μm sections, and the sections were mounted on silane-coated glass slides to minimize tissue loss through the staining process. Sections were maintained at room temperature for 12 h, then deparaffinized, and after hydration they were immunostained using an automated immunostainer (Ventana, Biotek Systems, AZ, USA). A 1:100 dilution of primary mouse monoclonal antihuman p75NGFR (sc-13577, Santa Cruz, CA, USA) was used to visualize p75NGFR expression. Primary antibody against p75NGFR was allowed to react at 35 °C for 32 min after blocking endogenous peroxidase activity with hydrogen peroxide. The glass slides were then treated with a biotinylated polyvalent secondary antibody solution, and sections were incubated with horseradish peroxidase-conjugated avidin–biotin complex followed by 3',3-diaminobenzidine and hydrogen peroxide. Finally, nuclei were counterstained with haematoxylin.

Other IAN tissues of 7 days after completing distraction were cut into 2-μm sections to observe the p75NGFR expression pattern under high magnification. These 2-μm tissue sections were immunostained for p75NGFR antibody using the above-described staining protocol.

Double immunofluorescent staining

Double immunofluorescent staining for NGF and p75NGFR was conducted in a dark room. Paraffin blocks were sectioned at 4 μm, and sections were mounted on silane-coated glass slides, as described above. Deparaffinization and antigen retrieval were performed simultaneously using Tris-EDTA buffer (LabVision, UK). Glass slides were incubated in a PTmodule™ (LabVision) at 100 °C for 25 min, washed with TBS (Tris-buffered saline, LabVision) for 2 × 3 min, and hydrogen peroxidase was applied at room temperature for 10 min. The slides were then washed with TBS solution, as described above. Rabbit polyclonal antihuman NGF (1:50, sc-548, Santa Cruz, CA, USA) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat polyclonal anti-rabbit IgG (1:50, Zymed, CA, USA) were selected as primary and secondary antibodies for the immunofluorescent visualization of NGF expression. Initially, primary NGF antibody was treated for 60 min at room temperature, slides were washed with TBS, and then the secondary antibody (TRITC) was treated for 60 min at room temperature and slides were rewashed with pure water. Mouse monoclonal antihuman p75NGFR (1:50, sc-13577, Santa Cruz) and fluorescein isothiocyanate (FITC)-conjugated goat polyclonal anti-mouse IgG (1:50, Zymed) were selected as primary and secondary antibodies for the immunofluorescent visualization of p75NGFR expression, and were sequentially treated for 60 min at room temperature as described above. Santa Cruz Biotechnology claims that these two primary antibodies (sc-548 and sc-13577) exhibit no cross-reactivity. The glass slides were then washed with pure water, and counter stained with 4′,6-diamidino-2-phenylindole (DAPI; VECTASHIELD®, Vector Lab., CA, USA). Slides were then cover slipped and immunofluorescent staining was observed under a fluorescent microscope.

Four controls were used to confirm the specificity of the immunoreaction involving the exclusion of primary or secondary

antibodies, or use of an inappropriate secondary antibody in the above-described immunostaining protocol. Control sections were processed in parallel with experimental tissue.

Histological evaluation

p75NGFR expression was assessed under an optical microscope. Two experienced pathologists, unaware of staining and stage details, evaluated the immunohistochemical staining patterns. A minimum of three sections per animal were evaluated at a time to determine protein expression. Tissue slices were analysed for antibody deposition in cellular components, such as Schwann cells and axons. Using the methods reported by TAVAKOLI et al.[24] and PARK et al.[19], intensities of positive immunostaining were graded as +++, ++, + and − for strong, moderate, weak and negative staining, respectively, and ± was used to represent focal and questionable weak staining.

The double immunofluorescent staining of NGF and p75NGFR was assessed by fluorescent microscopy (LEICA DM 6000B, Leica™, Germany) using a fluorescent digital camera (LEICA DC 500, Leica, Germany). Photographs were assessed using image analysis software (LEICA CW 4000, Leica). Co-expression was graded as positive (+) and negative (−) by the two experienced pathologists; ± was used to represent focal and questionable weak double staining.

Results

The results of mandibular DO have been reported[19]. DO proceeded smoothly without postoperative infection or failure, and the average mandibular lengthening was 8.7 ± 0.9 mm.

Immunohistochemical expression of p75NGFR

p75NGFR was not detected in non-distracted control IANs (Fig. 1A), but at 7 days after completing distraction it was clearly detected in distracted IANs (Fig. 1. B). Under high magnification of 2-μm tissue sections, p75NGFR expression was found to be greater in Schwann cells located in the outer layer of axons at 7 days after distraction (Fig. 1C). At 14 days after distraction, p75NGFR was moderately expressed in the distracted IAN (Fig. 1D). At 28 days after distraction, p75NGFR expression was significantly reduced, and was almost undetectable in the cellular components of IAN, except for focal expression on the perineurium (Fig. 1E). At 56 days after distraction, p75NGFR expression was not detected in the distracted IAN tissues (Fig. 1F). The patterns of p75NGFR expression in cellular components of the distracted IANs are summarized in Table 1.

Table 1. Semi-quantitative analysis of p75NGFR staining and positive double immunofluorescent staining for NGF and p75NGFR in the IAN after mandibular DO

	p75NGFR		NGF + p75NGFR
	Schwann cells	Axons	
Control	−	−	−
7 days after DO	+++	±	+
14 days after DO	++	−	+
28 days after DO	−	−	±
56 days after DO	−	−	−

Double immunofluorescent staining of NGF and p75NGFR

The co-expression of NGF and p75NGFR was not detected in non-distracted control IANs (Fig. 2A). At 7 and 14 day after completing distraction, their co-expression was detected in distracted IANs (Fig. 2B,C). At 28 days after distraction, definitive co-expression was not observed, except for some focal or questionable expression (Fig. 2D). At 56 days after distraction, no positive immunoreactivities for NGF and p75NGFR were observed in distracted IANs (Fig. 2E). The co-expression patterns of NGF and p75NGFR are summarized in Table 1.

Control slides, prepared by omitting primary or secondary antibodies, did not show any specific signals except for DAPI counter staining.

Discussion

In a previous report[2], the advantages of a dog model of mandibular DO were described. This model was the first used to examine mandibular DO and the surgical protocol and results have been relatively well described. Dogs are easily handled and are large enough to survive the surgical procedure. In addition, a commercial mandibular distractor can be placed in a dog's mandible without requiring adjustment.

In newborn rats, levels of NGF mRNA and NGF receptor (NGFR) mRNA in the sciatic nerve are 10 and 120 times higher, respectively, than in adult animals[6]. NGFR mRNA levels decreased steadily from birth and approached adult levels by the third postnatal week, whereas NGF mRNA levels decreased only after the first postnatal week and reached adult levels by the third week[6]. NGF mRNA is normally present at low concentrations in adult peripheral nerves, but when these nerves are damaged NGF mRNA levels rapid increase within 6 h in the Schwann cells, reaching 10-15 fold baseline levels within 24 h. Subsequently, NGF mRNA levels gradual increase and are maintained in an elevated state at 2 weeks after nerve injury[5,14]. The initial rapid increase in NGF mRNA in lesioned nerves is believed to occur due to an inflammatory response mediated by interleukin-1β from macrophages[6,15], whereas other inflammatory agents could mediate the increase of these neurotrophin mRNAs during the later chronic phase after nerve injury[16].

Low-affinity NGF receptor (p75NGFR) is undetectable in intact adult peripheral nerves, but its expression increases remarkably following nerve injury[4]. In an animal study, p75NGFR was found to be increased by at least 50-fold at 7 days after sciatic nerve transection[23]. In the absence of nerve regeneration, elevated NGFR mRNA levels are maintained for at least 20 days in both proximal and distal segments, but when nerve fibre regeneration was permitted, NGFR mRNA levels fell continuously[6]. In contrast to the effect of macrophage on NGF mRNA, no temporal changes were observed for NGFR mRNA, which means that NGF mRNA and NGFR mRNA regulatory mechanisms differ, i.e. NGF mRNA is regulated by infiltrating macrophages, whereas NGFR mRNA is regulated by contact between Schwann cells and axons[6,22].

During peripheral nerve regeneration, Schwann cells are over generated, but only one of four or six Schwann cells participates in the remyelination of damaged axons, and the excess show severe atrophy and gradually decrease in number[21]. HIRATA et al.[7] reported that severely atrophied supernumerary Schwann cells are eliminated by apoptosis during remyelination. p75NGFR was suggested to modulate both Schwann cell differentiation and apoptosis during nerve regeneration, and these different actions were related to the expression level of p75NGFR[8]. In other words, immediately after nerve damage,

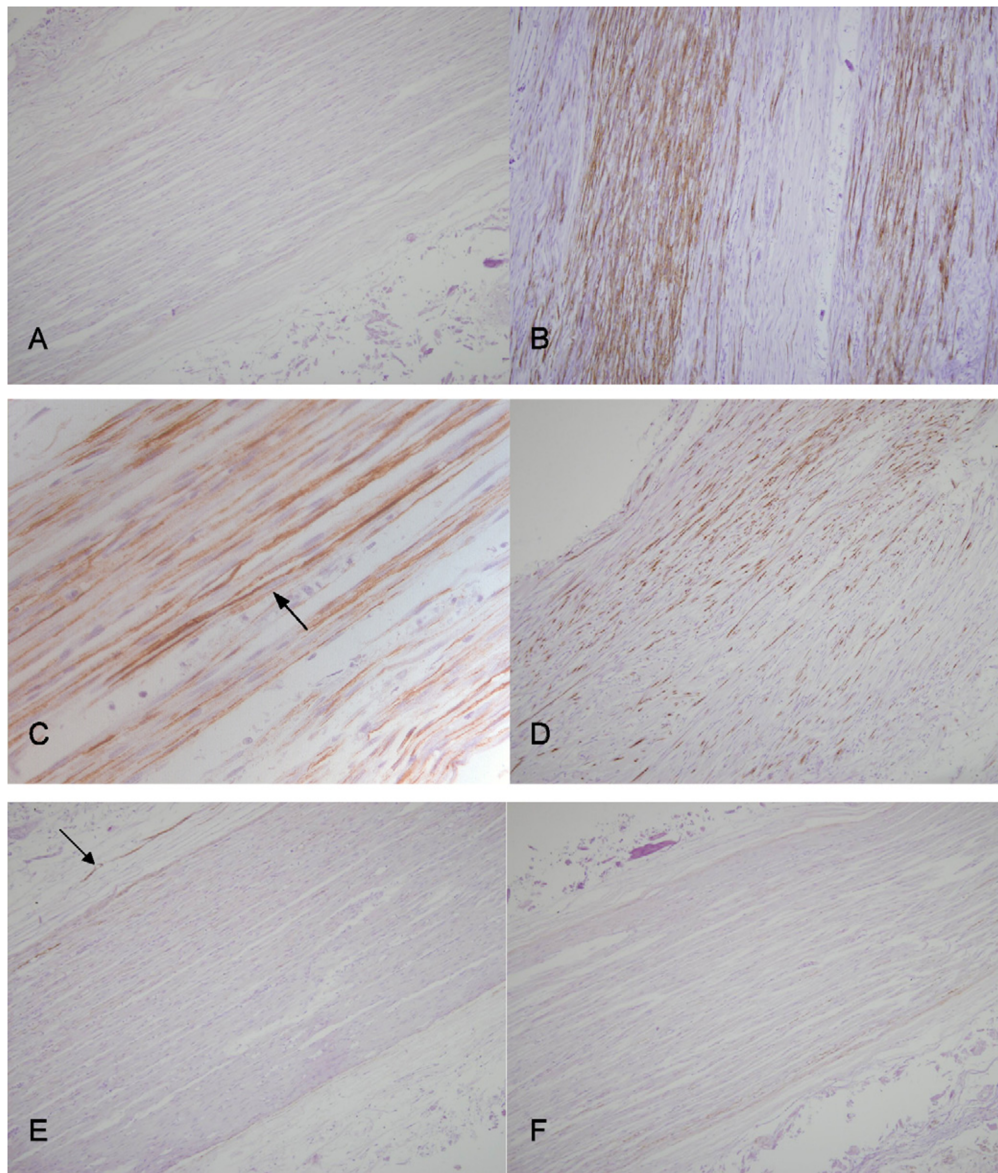

Fig. 1. Immunohistochemical expression of p75NGFR in the IAN after mandibular DO. (A) p75NGFR was not expressed in the non-distracted control IAN (x100 magnification). (B) At 7 days after distraction, strong p75NGFR staining was observed in the distracted IAN (x100). (C) Under high magnification of 2-μm tissue section, p75NGFR was found to be strongly expressed in the outer layer of axons (arrow) (x400). (D) At 14 days after distraction, p75NGFR was moderately expressed in distracted nerve tissues (x100). (E & F) No positive staining was seen in distracted nerve tissues, with the exception of some perineurium (arrow), at 28 and 56 days after distraction, i.e. p75NGFR expression had returned to baseline level of control nerve (x100).

NGF induces Schwann cell differentiation and proliferation through acting with rapidly increased p75NGFR. About 1 month after nerve injury, NGF induces supernumerary Schwann cell apoptosis through acting with rapidly decreased p75NGFR[26]. These two distinct functions might be understood as a mechanism benefitting nerve repair and regeneration.

Several researchers[3,10,19] have described the upregulation of various neurotrophic factors and related proteins in IANs after mandibular distraction, but studies of the relationships between neurotrophin receptors and nerve repair mechanisms after DO are rare. In the present study, it was observed that p75NGFR was up-regulated in IANs at 7 and 14 days after distraction, and this returned to baseline at 28 days after distraction. In a previous study[19], it was also observed that NGF expression returned to baseline in IANs at 56 days after distraction. These results concurred with those of another animal study[6], that the return of NGFR mRNA to normal levels started earlier than that of NGF mRNA, and the expression levels of NGF mRNA were greater in extent than those of NGFR mRNA after peripheral nerve transection. In addition, in the present study, strong p75NGFR expression was observed in Schwann cells located in the outer layer of axon. This result indicates that p75NGFR is expressed in the outer layer of damaged axons, and it is surmised that its expression is associated with remyelination via its interaction with various neurotrophins.

The autocrine growth activity for NGF has been studied in various fields, e.g. rat somatosensory cortex[20], rat trigeminal

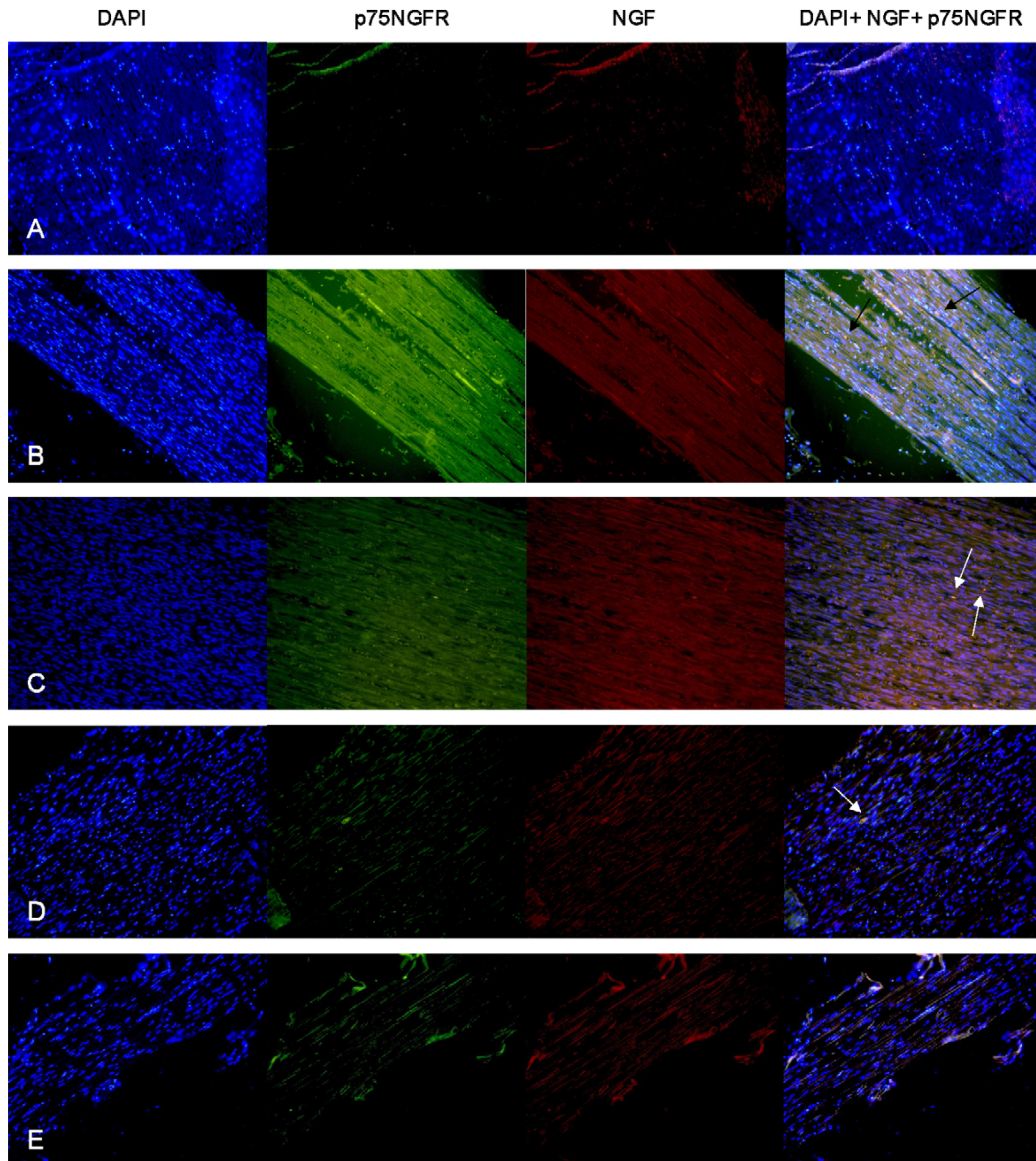

Fig. 2. Identification of cells immunoreactive for NGF and p75NGFR in IANs (DAPI, immunofluorescent expression of DAPI staining; p75NGFR, immunofluorescent expression of FITC staining; NGF, immunofluorescent expression of TRITC staining) (original magnification x200). (A) Positive double immunofluorescent staining for NGF and p75NGFR was not detected in the non-distracted control nerves. (B) At 7 days after distraction, NGF and p75NGFR double labeling was clearly observed in the distracted IAN (black arrows). (C) At 14 days after distraction, positive double immunofluorescent staining was maintained (white arrows). (D) At 28 days after distraction, although focal or weak positive double immunofluorescent expression was observed (white arrow), it did not justify a finding of definitive positive double staining in most nerve tissues. (E) At 56 days after distraction, no positive co-expression for NGF and p75NGFR was observed in the IAN.

system[12], B lymphocytes[25], malignant melanoma[17] and vascular smooth muscle cells[18]. In addition, Taniuchi et al.[23] reported nerve growth factor receptor induction in Schwann cells after experimental rat sciatic nerve axotomy. In the present study, the double immunofluorescent study demonstrated the co-expression of NGF and p75NGFR in distracted IANs at 7 and 14 days after distraction, which suggests that NGF is an autocrine growth factor in distracted IANs during the early consolidation period, and that it might contribute to the early recovery of IANs from damage induced by mandibular DO.

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