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Leading Research Paper
Distraction Osteogenesis

Expression of nerve growth factor and vascular endothelial growth factor in the inferior alveolar nerve after distraction osteogenesis

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Abstract. The objective of this study was to evaluate changes occurring in the inferior alveolar nerve (IAN) subsequent to mandibular distraction osteogenesis, with regard to the expression of nerve growth factor (NGF) and vascular endothelial growth factor (VEGF). Unilateral mandibular distractions (0.5 mm each, twice per day for 10 days) were conducted on 8 mongrel dogs. Two animals were killed at 7, 14, 28 and 56 days after completion of distraction. The distracted IAN and contralateral control nerve were then harvested and analysed histologically and immunohistochemically. Signs of acute nerve injury, including demyelination, were observed in the distracted IAN on the 7th and 14th day after distraction. At 56 days, the histological features of the distracted IAN were similar to those of the control nerve. The levels of NGF and VEGF expression were significantly elevated on the 7th and 14th day after distraction. NGF was expressed in most of the distracted nerve tissues, but VEGF was primarily detected in Schwann cells and the neurovasorum. VEGF expression had returned to normal but NGF expression was still profoundly elevated 28 days after distraction. NGF expression returned to normal levels at 56 days after distraction. NGF and VEGF appeared to have been elicited from the Schwann cells and damaged nervous tissues, and they may play important roles in the initial healing of damaged nerves. VEGF expression returned to normal more quickly than did NGF expression. This may indicate that hypoxic conditions within the distracted nerve had recovered to normal during the early stages of consolidation. Micro-vessels in the distracted nerve may have recovered more rapidly than did the nerve tissue itself.

Key words: distraction osteogenesis; inferior alveolar nerve; nerve growth factor; vascular endothelial growth factor.

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Distraction osteogenesis (DO) has become a frequently used technique in the reconstruction of bony defects induced by tumours, deformity and trauma to the maxillofacial region. The clinical application of DO began to gain attention after the Russian physician, Ilizarov[11], successfully employed the technique for the lengthening of limbs in the 1950s. In his study, it was determined to be most effective to distract the leg at a rate of 1 mm/day in 4 equal increments of 0.25 mm each[11]. Clinically, McCarthy et al.[17] reported the first cases of gradual distraction of the human mandible. They reported successful mandibular lengthening from 18 to 24 mm, over a period of 75 months, in a case series of 4 children.

Although, a great many experimental and clinical studies on DO have been conducted, changes in the surrounding tissues, including nerve or vascular tissues, remain poorly understood. One of the ramifications of our lack of understanding is the continuing existence of associated complications, including injuries to nerve fibres after distraction. Some clinicians have detected neurosensory disturbances in up to 30% of patients who have undergone limb-lengthening procedures[1,6]. Higher sensory disturbances have also been reported in patients undergoing mandibular distraction procedures[10]. The frequent incidence of sensory disturbances following mandibular DO has been generally attributed to the position of the inferior alveolar nerve (IAN) bundle within the mandibular canal, which allows for stress during distraction[10].

In several previous animal studies[9–11], Wallerian degeneration has been shown to occur in the IAN following gradual mandibular distraction, but the recovery process, which involves remyelination or regeneration, has also been shown to occur simultaneously. Schwann cells and several neurotrophic factors are crucially important to this process of nerve regeneration[10]. Of these neurotrophic factors there are: first, the neurotrophins, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF); second, the neurokines, including ciliary neurotrophic factor and leukaemia inhibitory factor; and third, transforming growth factor β and glial cell line-derived neurotrophic factor[5]. Recently, several studies[19,21,22] have suggested that vascular endothelial growth factor (VEGF) is also involved in promotion of the growth of Schwann cells, which in turn boosts axon growth, thereby sparking interest in the possible neurotrophic effects exerted by VEGF. The objective of this study was to determine changes occurring in the IAN subsequent to mandibular distraction in dogs, by the examination of nerve tissues and measurement of the activities of NGF and VEGF during nerve remodelling following the distraction.

Materials and methods

Eight mongrel dogs, each between 1 and 2 years of age and weighing about 10 kg, were used in this study. Animal care was consistent with the guidelines provided by the Animal Center for Medical Experimentation at Gyeongsang National University.

Surgical protocol

Animals were anaesthetized via intravenous injection of a mixture of 10 mg/kg ketamine (Ketalar, Yuhan Corp., Korea) and 2.0 mg/kg 2% xylazine (Rompun, Bayer Korea, Korea). The surgical fields were sterilized with betadine solution, and then 2% lidocaine HCL with 1:100,000 epinephrine was injected into the right submandibular skin. After sequential dissection of the submandible, buccal and lingual corticotomies were conducted between the 3rd and 4th premolars, or between the 4th premolar and the 1st molar. Care was taken to ensure the anatomical integrity of the IAN. The intraoral mandibular distractor (Leibinger, Germany) was then positioned on the buccal cortical bone, after the mandible had carefully been fractured in a linear manner. To prevent any damage to the IAN, the cortical screw was positioned as closely as possible to the side of the alveolar bone (Fig. 1). The retromandibular skin was then perforated to expose the distractor rod. The wound was closed in 2 layers with 3-0 Vicryl to the platysma and 3-0 nylon to the skin. First generated cephalosporin (20 mg/kg; Cefazolin, Yuhan Corp., Korea) was then injected intramuscularly twice a day for 5 days after completion of surgery. After a 5-day latency period, the mandible was distracted for 10 days at a rate of 1.0 mm/day in 2 increments per day.

Specimen preparation

After the administration of general anaesthesia, 2 animals each were killed by KCl injection at 7, 14, 28 and 56 days after completion of distraction. Immediately after the animals were killed, the right mandibles were harvested *en bloc*, and the elongated segments of the IAN in the distracted callus were carefully dissected and separated (the distraction group). The left undistracted mandible was then also block-resected, via an identical procedure, after which the normal IANs were harvested (the control group). The harvested nervous tissues were then separated into 2 sections for immunohistochemical studies and light microscopic observation after the preparation of semithin (1-μm) sections.

One of the tissue specimens was immersed in 10% neutral buffered formalin for 24 h, then embedded into paraffin blocks for the immunohistochemical studies. In brief, the paraffin blocks were cut into 4-μm sections, and the sections were mounted on silane-coated glass slides to minimize tissue loss throughout the staining process. The sections were maintained at room temperature for 12 h, then deparaffinized. After hydration, immunostaining was conducted using an automated immunostainer (Ventana, Biotek Systems, Tucson, AZ, USA). A 1:100 dilution of primary rabbit polyclonal antihuman NGF (sc-548, Santa Cruz, CA, USA) and a 1:500 dilution of primary rabbit polyclonal antihuman VEGF (sc-507, Santa Cruz, CA, USA) were then utilized for the induction of NGF and VEGF expression, respectively. To enhance the immunostaining, the slides for VEGF were treated

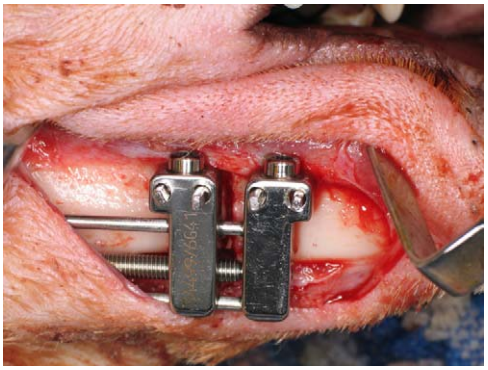

Fig. 1. Mandibular distractor positioned via submandibular approach.

with 0.1% trypsin solution (S2012, Dako, Denmark). They were incubated at 37 °C for 20 min in a humidity chamber. Primary antibodies against NGF and VEGF were then allowed to react at 35 °C after the blockage of endogenous peroxidase activity via the administration of hydrogen peroxide. After 32 min, the glass slides were treated with a biotinylated polyvalent secondary antibody solution. The sections were then incubated with horseradish peroxidase-conjugated avidin–biotin complex, followed by treatment with 3,3-diaminobenzidine and hydrogen peroxide. Finally, the nucleus was counterstained with haematoxylin.

The other specimens were cut into 1-mm^3 pieces to prepare the semi-thin sections. These sections were fixed in 2.5% glutaraldehyde solution (pH 7.4, 0.1 M cacodylate buffer, 4 °C) for 2 h, and then washed 3 times in cacodylate buffer. The sections were post-fixed in the same buffer with 1% osmium tetraoxide (OsO_4) solution for 2 h, dehydrated with ethanol, and embedded in an Epon mixture. The semi-thin sections were cut into 1-μm sections, and stained with 1% toluidine blue for observation under a light microscope at ×400 magnification.

Immunohistochemical expression was assessed under a light microscope. Two experienced pathologists, both of whom were 'blind' to the staining and stage, evaluated patterns of immunohistochemical staining. A minimum of 3 sections per animal were evaluated at each time point for each protein analysed. The slices were analysed essentially for antibody deposition in the cellular components, including Schwann cells, myelin sheaths and endothelial cells. According to the methods reported by Tavakoli et al.[23], the intensity of positive immunostaining was graded as +++, ++, + and − for strong, moderate, weak and negative staining, respectively. A grade of +/− was used to represent focal or questionable weak staining.

Results

DO proceeded smoothly in all of the animals, with no surgical infections or failures. The mandible was lengthened in all animals by a mean of 8.7 ± 0.9 mm, as determined by the changed distance between proximal and distal pins, which was measured immediately after distractor placement and before killing. New bone formation at the distracted mandible was observed by radiography, at 28 and 56 days after completion of distraction (Fig. 2).

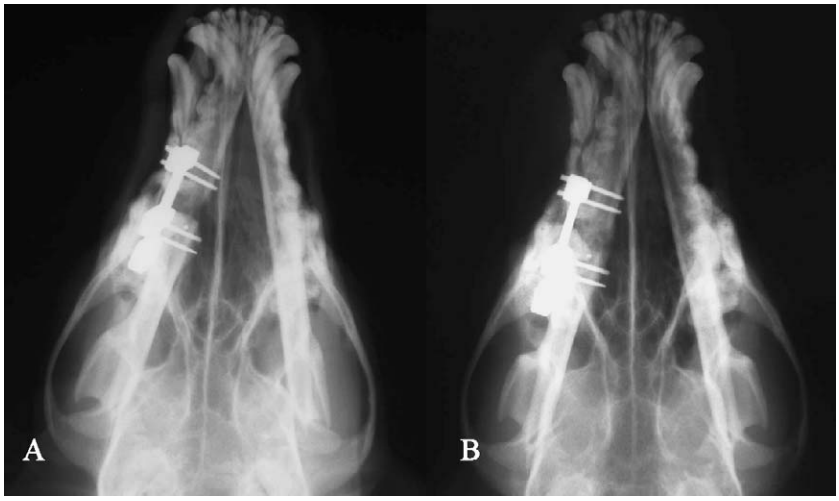

Fig. 2. Radiographs demonstrating the distraction and healing of the mandible. (A) Immediately after completion of distraction, showing a distraction gap. (B) At 28 days after distraction; note the bone consolidation within the gap.

Light microscopical observations

In the control IAN, large myelinated fibres were predominantly observed, along with some thin myelinated and unmyelinated fibres in the interstices of the large fibres. On the 7th day after distraction, extensive demyelination of the large myelinated nerve fibres was observed. At 14 days following distraction, there were no specific differences from what had been observed on the 7th day, but there were signs of remyelination, primarily in the slightly increased ratio of myelinated to non-myelinated fibres. At 28 days following distraction, much thicker myelin sheaths were observed, along with an increase in the number of large myelinated fibres. The thickness and density of the newly formed myelin fibres were, however, still fairly irregular. At 56 days after distraction, remyelination was extensive, resulting in a significantly increased ratio of myelinated fibres to non-myelinated fibres, as well as much thicker myelin sheaths (Fig. 3).

Immunohistochemical expression of NGF and VEGF

NGF was weakly expressed around the axon and the myelin sheath in the control IAN. At 7 days following distraction, the expression of NGF increased by a significant amount in almost all of the distracted nerve tissue, including the Schwann cells, myelin, axons and the endothelial cells in the neurovasorum. This intensified staining persisted through the 14th and 28th days after distraction. A dramatic reduction in the expression of NGF was observed, however, at 56 days, when it was expressed at levels roughly equal to those of the control nerves (Fig. 4).

VEGF was weakly expressed only in the endothelial cells of the neurovasorum, and no VEGF staining was observed in the other tissues of the control IAN. VEGF, however, was expressed abundantly in the Schwann cells and the disrupted nerve fibres at 7 days after distraction. On day 14, although VEGF was expressed only to a minor degree as compared with that observed 7 days after distraction, it was still fairly abundantly expressed as compared to the expression seen in the control nerve. VEGF expression was clearly detected not only in the vascular tissue, but also in the Schwann cells of the nerves on the distracted segment. At 28 and 56 days after distraction, the expression of VEGF was reduced significantly, with almost no remaining expression in the nerve tissues of the distracted segment. Nevertheless, it was still expressed, albeit weakly, in the endothelial tissues of the neurovasorum (Fig. 5).

The pattern of NGF and VEGF expression in cellular components of the distracted IAN is summarized in Table 1.

Discussion

Several studies have focused on nerve tissue damage occurring after DO. In a study conducted by Fink et al.[4] , demyelination was detected in both the peroneal and tibial nerves after 25 days of leg lengthening in dogs, which had been conducted at a rate of 1 mm/day. These morphological changes in the nerve fibres were, however, followed by a remyelination period, which commenced immedi-

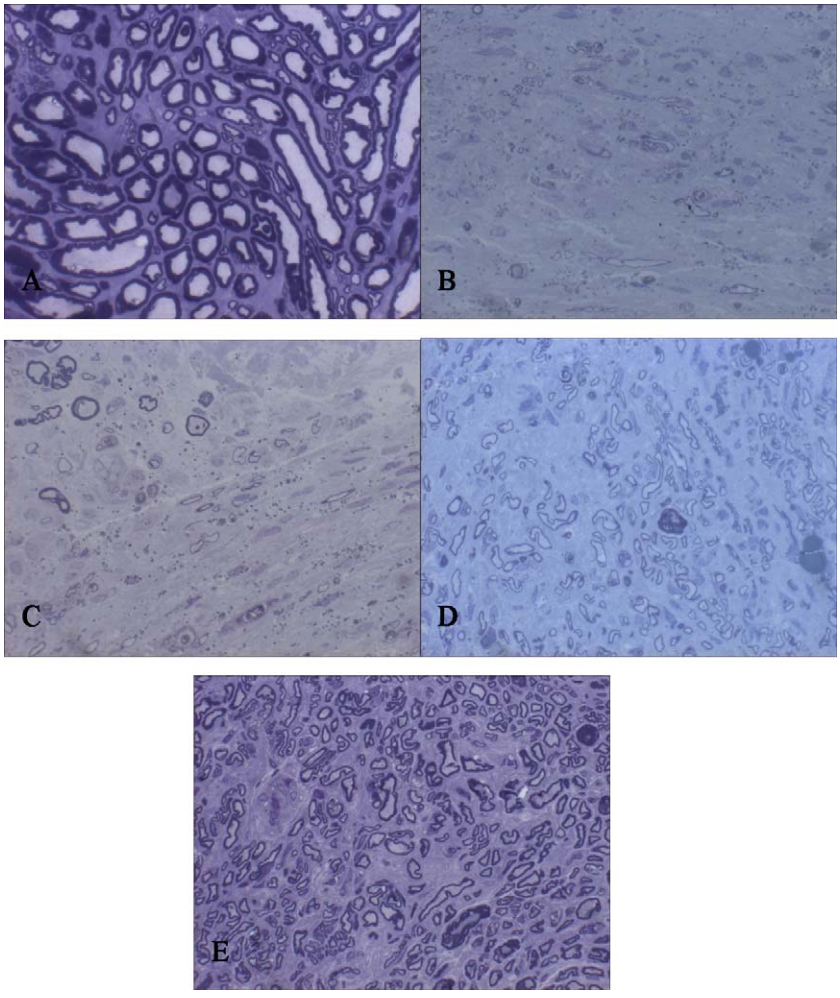

Fig. 3. Semi-thin sections (stained with toluidine blue) of the control and experimental inferior alveolar nerves (original magnification ×400). (A) Large myelinated fibres can be seen in the control IAN. (B) There is severe loss of large myelinated fibres, such that small myelinated and unmyelinated fibres are predominantly observed at 7 days after distraction. (C) At 14 days after distraction, partial remyelination and thickening of the myelin sheath can be seen. (D) At 28 days after distraction, progressive thickening of the myelin sheath is observed, and there is a return to almost normal levels of large myelinated fibres at 56 days after distraction (E).

ately after the infliction of the damage. Skoulis et al.[20] observed a distraction rate of 11% in the sciatic nerve after a 25-mm distraction of the rat femur. They detected morphological changes only in the distracted nerve itself in the groups with distraction rates of 0.5 mm/day and 1 mm/day, whereas they detected these morphological changes occurring throughout the entirety of the nerve in the group with a distraction rate of 1.5 mm/day. Such differences were consistent with the findings of other studies, in which the nerve fibres were straightened and then stretched along with the perineurium with increasing stretching[2], whereas nerve degeneration was observed in all nerve fibres when they were stretched to the limit of their elasticity[8]. Thus, to precisely assess distraction rates, the elastic properties of the nerve fibres clearly must be considered[20].

Table 1. Summary of semi-quantitative analysis of NGF and VEGF staining in cellular components of the IAN after mandibular DO

	Schwann cells		Myelin sheaths		Endothelial cells	
	NGF	VEGF	NGF	VEGF	NGF	VEGF
Control	+	–	+	–	+	+
7 days after DO	+++	+++	+++	+/–	+++	+++
14 days after DO	+++	+++	+++	+/–	+++	+++
28 days after DO	+++	–	+++	–	+++	++
56 days after DO	+	–	+	–	+	++

Fink et al.[4] determined that non-myelinated fibres are damaged to a somewhat greater degree than are myelinated fibres, suggesting that the myelinated sheath may provide some protection against tension-induced injury. Conversely, Hu et al.[10] and Wang et al.[24] reported that large-diameter myelinated fibres were most vulnerable to tensile strain while unmyelinated and small-diameter myelinated fibres remained intact after mandibular distraction, even when distraction was applied at a rate of 2 mm/day.

In this study, with regard to the semi-thin sections, demyelination and destruction of large myelinated fibres were determined to be extensive on day 7 after completion of distraction, and the structural changes in the nerve fibres continued until day 14, although some signs of remyelination were seen. This result is consistent with previous findings that nerve regeneration occurred approximately 2 weeks after surgery[9]. Nerve fibres suffering DO-induced damage thus began to regenerate within 2 weeks of the completion of distraction, and these fibres almost completely recovered their normal morphological appearances during the subsequent 8-week consolidation period.

To enhance peripheral nerve regeneration, 2 distinct methods have been developed: the manipulation of Schwann cells and the use of neurotrophic factors. Schwann cells are integral in the promotion of regeneration, and exert this effect via 3 distinct mechanisms: first, by increasing their rate of synthesis of cell-surface adhesion molecules (CAMs); second, by elaborating the basement membrane, which contains many extracellular matrix proteins and third, by generating a variety of neurotrophic factors and their receptors[5]. Among the neurotrophic factors, NGF is one of the best-known nerve-derived factors; it is a 26-kDa non-glycosylated, homodimeric polypeptide, and its activity is known to be crucial for the survival and differentiation of nerve cells. When the peripheral nerves were damaged, the Schwann cells generated NGF within 24 h, eventually effecting a 10–15-fold increase from baseline levels; this effect persisted for at least 2 weeks after the infliction of injury to the nerve[14]. These elevated NGF concentrations can be explained by the increased secretion of NFG in the Schwann cells and the release of interleukin-1 by the invaded macrophages within the damaged nerve segment[5,14,15].

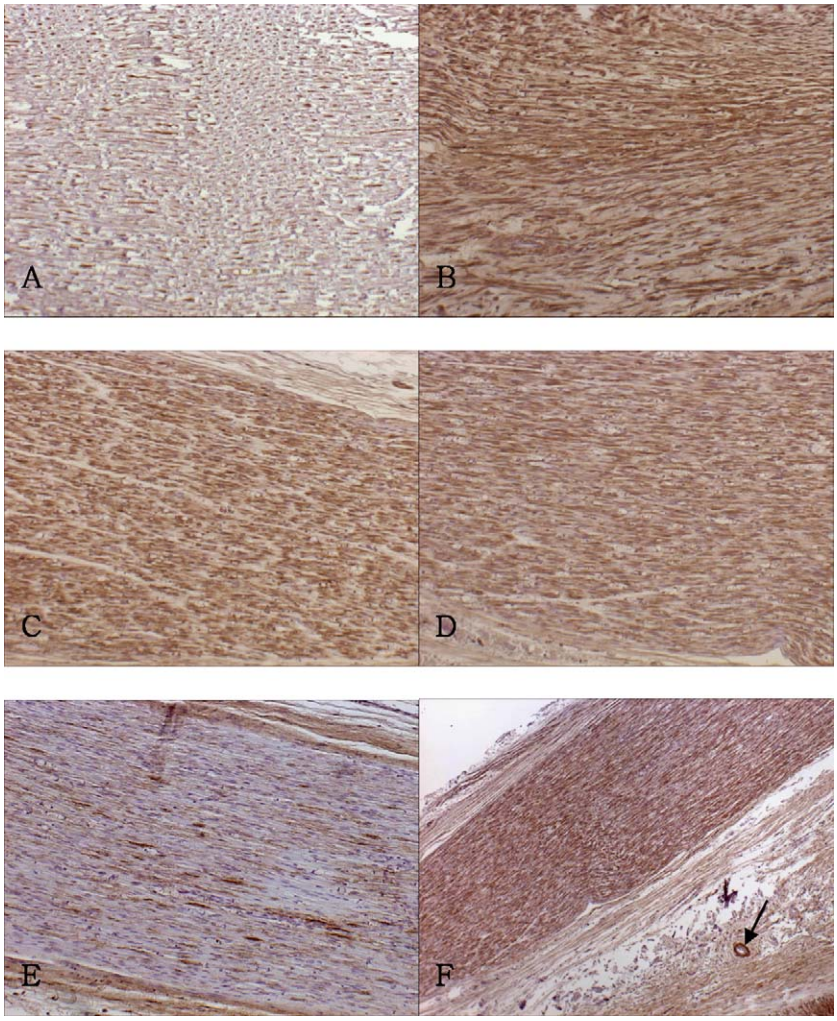

Fig. 4. NGF expression in the inferior alveolar nerve. (A) NGF expression is evenly and weakly distributed along the normal axons (×100 magnification). (B) At 7 days after distraction, NGF shows very strong staining in all the distracted nerve fibres, and this persisted for 14 and 28 days after distraction (C and D, ×100). (E) At 56 days, the levels of NGF expression were similar to those of the control specimens (×100). (F) The endothelial cells of the supportive neurovasorum of the IAN on the distracted side stained positively for NGF at 28 days after distraction (black arrow, ×40).

Farhadieh et al.[3] reported observing widespread expression of NGF and BNDF in the IAN after mandibular distraction. They attributed this to stimulation of Schwann cells by myelin sheath debris during the distraction procedure. That is to say, the proliferation of Schwann cells and increase in the concentration of NGF were triggered in response to signals from the myelin sheath debris at the time of the nerve damage, and the elevated NGF concentrations were maintained by the activity of macrophage-derived cytokines[15].

In this study, the expression and secretion of NGF were found to effect a substantial increase in the number of Schwann cells and damaged nerve tissues, including myelin sheath, for 4 weeks after distraction, although these levels returned to normal sometime between weeks 4 and 8. It might be suggested that the damaged nervous tissues were actively recovered until that period. This, however, result was not consistent with the findings of other studies, in which the increased NGF levels persisted for approximately 2 weeks before beginning a gradual decline[14]. This discrepancy with regard to duration is likely to vary with the nature and severity of the nerve damage. Simple nerve damage might be regenerated after about 2 weeks, but DO-induced IAN damage required a remodelling time of over 4 weeks, therefore indicating an increase in the expression and secretion of NGF until that time had elapsed.

Blood flow in the lengthened segment also appears to be an important factor influencing nerve tissue damage following DO. Ogata & Naito[18] asserted that blood flow might be completely discontinued in cases in which the nerve tissue was distracted by over 15%, thereby inflicting critical damage to the nervous system. Lundborg & Rydevik[16] report similar findings. Unlike these studies, however, Ippolito et al.[12] claimed that DO-induced changes in the nerve tissue were more profoundly associated with the elasticity of tissue than with the disturbance of blood flow, as nerve fibres are more susceptible to damage than is vascular tissue. Thus, slow and gradual distraction might cause only minimal damage to nerve tissues.

Recently, a host of studies have focused on the role of VEGF as a component of research into changes in blood flow after the infliction of nerve damage. VEGF is a 46-kDa heparin-binding homodimeric glycoprotein that is known to be structurally related to platelet-derived growth factor. VEGF has been shown to be activated under hypoxic conditions, and has also been demonstrated to stimulate angiogenesis[19,22]. In addition, VEGF is a neurotrophic factor, as well as a neuroprotective factor, and can stimulate the regeneration of nerves[13,19,21]. Increased VEGF levels stimulate angiogenesis in association with the endothelial flk-1 receptor, and also have a neuroprotective and neurotrophic function in association with the neuronal flk-1 receptor[22]. Gupta et al.[7], in a series of animal studies, noted a significant increase in the expression of VEGF in Schwann cells after application of nerve compression. The expression of VEGF began to increase at 2 weeks after compression, achieved peak levels at 1 month, and remained strong for about 6 months. Sondell et al.[21,22] reported that VEGF treatment promoted survival of both neurons and Schwann cells in adult ganglia. These effects of VEGF were distinctly different from those of NGF, which primarily increases the number of regenerating axons[21].

In this study, expression of VEGF in the distracted nervous tissues was shown to have increased markedly immediately after DO, and then returned to its normal morphological appearance between 2 and 4 weeks after completion of distraction, in contrast to the results of Gupta et al.[7], described above. The primary reason for this discrepancy probably lies in the different experimental methods used, i.e. the hypoxic condition in distraction-induced nerve damage recovered more quickly than in cases of compression-induced nerve damage. Also, the expression of VEGF was clearly detected in Schwann cells, as in the other study[7], meaning that Schwann cells were the main source of VEGF.

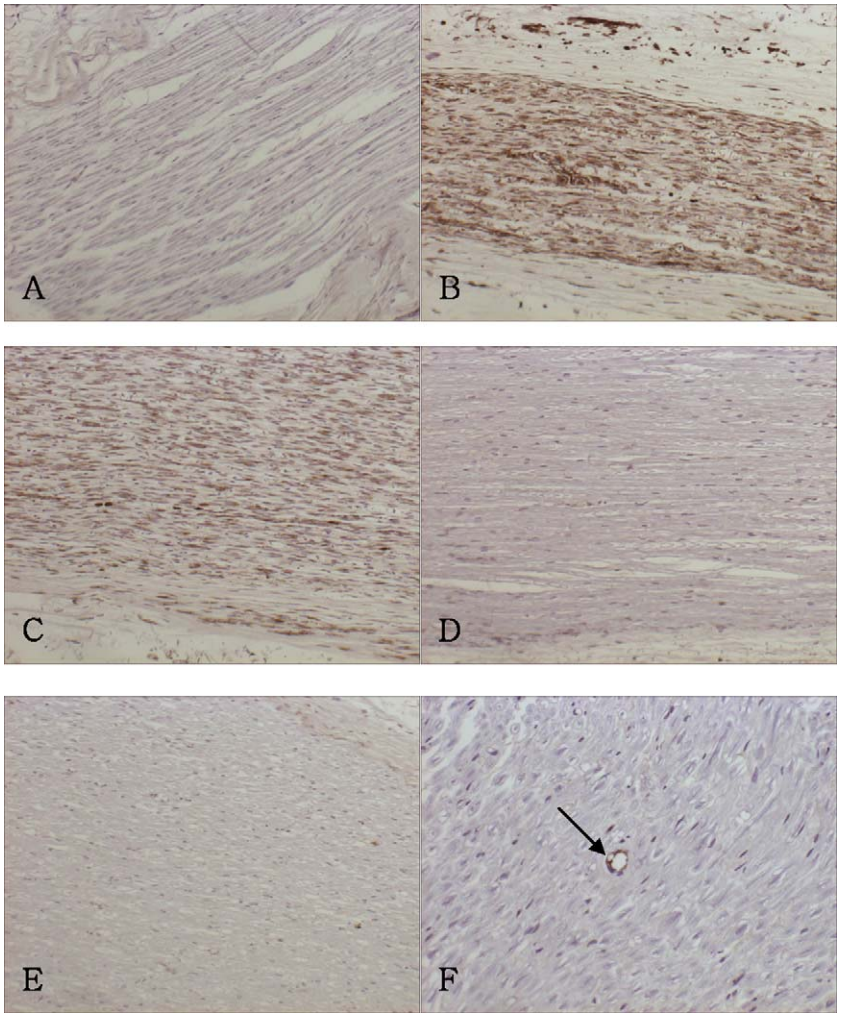

Fig. 5. VEGF expression in the inferior alveolar nerve. (A) VEGF is not expressed in the control inferior alveolar nerve (×100 magnification). (B) At 7 days after distraction, VEGF staining was strong in the Schwann cells and disrupted nerve fibres (×100). (C) At 14 days after distraction, although VEGF is expressed weakly compared to that observed 7 days after distraction, it is still expressed fairly strongly as compared to the control nerve (×100). (D and E) No positive staining was seen in distracted nerve tissues with the exception of the endothelial cells at 28 and 56 days after distraction; therefore the expression levels of VEGF are similar to the levels of the control nerve (×100). (F) Black arrow indicates positive expression in endothelial cells of the supportive neurovasorum at 56 days after distraction (×200).

The results of the present study indicate that NGF returned to normal levels on the 56th day after surgery, whereas VEGF returned to normal levels on day 28. Considering the fact that VEGF levels increase under hypoxic conditions, this would appear to suggest that hypoxic conditions within the distracted nerve had returned to normal during the early consolidation period. It is, therefore, likely that micro-vessel recovery takes place more rapidly than does nerve remodelling in the distracted nerve. To confirm these results, further studies will be required, and should include many different VEGF receptors.

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