Locally Applied Nerve Growth Factor Enhances Bone Consolidation in a Rabbit Model of Mandibular Distraction Osteogenesis

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ABSTRACT: Distraction osteogenesis is widely used in treating deformities, defects, and fractures of both long bone and craniofacial bone. Demands for acceleration of bone consolidation are increased in distraction osteogenesis. It has been shown that nerve growth factor (NGF) can enhance innervation and bone regeneration in a fracture model and stimulate differentiation of osteoblastic cells. In this study, we tested the ability of locally applied NGF to enhance bone regeneration in a rabbit model of mandibular distraction osteogenesis. Twenty rabbits underwent bilateral distraction osteogenesis with a rate of 0.5 mm per 12 h. Two times 0.04 mg hNGF in buffer were injected into callus after the end of distraction. The contralateral side received placebo injections. Rabbits were euthanized at consolidation time of 14 and 28 days. Specimens were subjected to radiography, callus dimensions measurement, mechanical testing and bone histological and histomorphometric analysis. The maximum load, bone volumetotal volume, mineral apposition rate of the 1st to 11th day, and mineralized bone percentage were significantly higher in the hNGFβ side at 14 and 28 days (p<0.05). The data indicate that locally applied hNGFβ can accelerate callus maturation and may be an option to shorten the consolidation period in distraction osteogenesis. © 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:2238-45, 2006

Keywords: nerve growth factor; distraction osteogenesis; bone regeneration; rabbit

INTRODUCTION

Distraction osteogenesis (DO) has become a widely accepted method in orthopedic surgery and has been applied to resolve clinical problems such as deformities, defects and fracture non-unions of long bone.1,2 DO has been enthusiastically applied to craniofacial deformities including hemifacial microsomia,3 cleft palate,4 and pediatric craniofacial syndromes, such as sleep apnoea.5 However, the long period of bone consolidation time can contribute to complications, such as bone infection, pain and fracture of distraction device. Consequently, the need exists for acceleration of bone consolidation during DO.

Sensory and sympathetic nerves play an important role in normal skeletal metabolism and fracture repair.6,7 In sympathectomized animal models, bone resorption is increased and bone deposition and mineralization are decreased.8,9 Additionally, sympathetic nerves can also result in osteoporosis in mandibular bone.10 Nerve growth factor (NGF) is an important neurotrophin involved in development, maintenance and regeneration of sensory and sympathetic nerves.11,12 In vitro studies have demonstrated that NGF can stimulate differentiation of nerve cells.13,14 Therefore, NGF may play a crucial role in bone regeneration. In a rabbit inferior alveolar nerve defect model, an incidental observation revealed that administration of NGF stimulated new bone formation around the induced regenerating axons.15 In a rat fracture model, local administration of NGF increased the quality and rate of fracture repair.16 Additionally, NGF enhanced new bone ingrowth to a collagen/hydroxyapatite composite.17 In light of these findings, local administration of NGF is likely to be useful for bone regeneration in DO.
Expression of growth factors is closely related with strain in DO. For example, strong expression of angiogenic factors such as VEGF and bFGF, BMPs and their receptors was found during the phase of active distraction, and then much weaker expression was observed when distraction was discontinued.\(^{18-20}\) Expression of NGF in distracted inferior alveolar nerve was stimulated by strain and decreased in consolidation period.\(^ {21,22}\) In a fracture healing model, expression level of NGF mRNA was increased right after fracture, peaked at 2 days after fracture and began to decrease thereafter.\(^ {23}\) The expression of NGF in distracted bone tissue has not been settled yet, but it is very likely to mimic the expression profile of BMPs. Exogenous NGF may therefore play a role in acceleration of callus maturation at the beginning of consolidation period.

To our knowledge, no one has investigated the effects of NGF on bone formation during distraction osteogenesis. Therefore, the objective of this study is to determine the ability of locally applied NGF to enhance bone regeneration during mandibular DO in a rabbit model.

**MATERIALS AND METHODS**

**Experimental Animal Model**

Twenty skeletally mature (3.1-3.5 kg), male, New Zealand White rabbits were studied. The animals were housed and cared for in accordance with the guidelines established by Animal Center for Medical Experiment at Fourth Military Medical University. Rabbits were anesthetized with an intramuscular injection of 50 mg/kg ketamine hydrochloride and an intravenously injection of 40 mg/kg Phenobarbital sodium. 1% lidocaine with 1:200,000 epinephrine was administered subcutaneously in the submental triangle. The incision was made in the midline, and periosteal flaps were reflected laterally to visualize mental nerve anterior to the first premolar teeth. A vertical osteotomy was performed bilaterally between the premolar teeth and mental foramen using a fissure bur. A custom-made distraction device (Zhongbang Titanium Biomaterials Corporation, Xi'an, P.R. China) was fixed with the distraction rod emerged into the labial vestibule (Fig. 1). The titanium device traveled 0.4 mm per complete rotation. Four microplates were secured to the mandible with self-tapping tapered screws (1.5 mm in diameter and 7 mm in length). All 10 screws were placed perpendicular to the lateral aspect of the mandible, parallel to each other and across both cortices. The clap-shaped microplates provided additional support for retention of the device. The osteotomy was completed with a fine chisel. Great care was taken to avoid injury to the inferior alveolar nerve, and lingual periosteum was left intact. The wound was then closed in layers. The upper incisor teeth were burred to approximately half of their projecting distance, in order to reduce the impact on the lower gingival due to induced malocclusion. Postoperatively, the rabbits' clinical condition, dietary habits and weight were monitored. After
Histology and Histomorphometry

a latency period of 3.5 days, gradual distraction was performed at a rate of 0.5 mm per 12 hours for 10 days.

NGF Administration and Fluorochrome Labelling

On the 1st and 3rd days after the end of distraction, two times 40µg hNGFβ (Laboratory of biochemistry, Fourth Military Medical University, Xi’an, P.R. China) in 0.15ml isotonic saline were injected into the palpable callus at the treatment side percutaneously. The contralateral side served as control and received of placebo (isotonic saline) simultaneously in an identical manner. A sequential intravital fluorochrome labelling was performed in all the animals by intramuscular injection according to the following scheme: in the 14 days consolidation experiment, 30mg/kg tetracycline hydrochloride on the 1st and 11th days after the end of distraction; in the 28 days consolidation experiment, 30 mg/kg tetracycline hydrochloride on the 1st and 22nd days and 90 mg/kg calcine green on the 11th day after the end of distraction.

Specimen Processing and Mechanical Testing

The rabbits were sacrificed at consolidation time of either 14 (n=8) or 28 (n=8) days. Under general anesthesia, both carotid arteries were perfused with normal saline to remove blood, followed by 2.0% paraformaldehyde and 2.5% glutaraldehyde in 0.1M/L PBS (PH7.4) for the internal fixation of the tissue. The entire mandible was harvested and the soft tissue was excised. The amount of distances achieved was measured using a sliding caliper (mean of superior and inferior border). Total regenerate callus dimensions measurement was performed by measuring the external labial-lingual and superior-inferior dimensions at the middle of the distraction callus using a sliding caliper. Lateral X-rays were performed and the radiographs of regenerated callus were graded by comparing them with aluminum plates with a gradient of thickness (20 steps, 0.5 mm/step).

Both treatment and control sides were evaluated for twice with a 3-day interval by a single, experienced examiner who was blinded to the experimental sides. A difference of more than or equal to 0.5 mm of aluminum thickness was considered as a difference in radiodensity. Hemimandibles were subjected to a three-point bending test using an AGS-10KN materials testing machine (Shimadzu Corporation, Kyoto, Japan) at a displacement rate of 0.5 mm/s. The maximum load was obtained for all specimens. The distracted region, including 1-3 mm of neighboring normal bone, was cut from each hemimandible, and immersed in the fixation solution described above for additional 24 hours at 4°C.

Histology and Histomorphometry

After postfix, the specimen was cut into two parts in the axial plane. The upper half was dehydrated and embedded in methyl-methacrylate. 30-µm thick sections were cut for fluorescence measurements using a DM IRB fluorescence microtome (Leica, Germany), and the mineral apposition rates of the 1st-11th and 12th-22nd days of consolidation period were determined by measuring the distance between the labeled zones. Undecalcified sections were also stained with von Kossa for evaluation of mineralized bone percentage. The lower half of the specimen was decalcified in buffered 14.5% EDTA (PH7.3) for 20-30 days, dehydrated and paraffin embedded. Each block was cut in 5-µm thick in the axial plane and stained with hematoxylin and eosin.

Bone histomorphometric analysis was performed on four sections for each sample using a NIH Image Analysis System. Eight images were randomly selected from each section and measured for twice with a 3-day interval by a single, unbiased examiner who was blinded to the experimental sides. Large basophilic cytoplasm and eccentrically placed nucleus were recognized as osteoblastic morphology. The related bone histomorphometric parameters included bone volume/total volume (BV/TV, %, ratio of mineralized and un-mineralized bone volume to the total tissue volume of distracted region), number of osteoblasts (µm², number of active osteoblasts per area, excluding lining cells) and mineral apposition rate (µm/day, distance between labels per labelling period).

Statistical Analysis

The results were presented as mean and standard error of the mean. A paired t test was used to calculate differences between the treatment and control sides in callus dimensions, maximum load and bone histomorphometric parameters. p≤0.05 was considered statistically significance.

RESULTS

Four of the 20 rabbits had to be excluded from the study. One animal died during operation because of anesthesia problem. Two animals developed infection of the wounds and one respiratory infection. The remaining 16 rabbits showed good tolerance to the whole experimental procedure and were divided into two separate groups with consolidation time of 14 (n=8) and 28 (n=8) days respectively. All specimens of the 16 rabbits underwent radiographic evaluation, callus dimensions measurement, mechanical testing and decalcified and undecalcified histomorphometric analysis. No difference on the amount of distances achieved or regenerate callus dimensions was observed between the treatment and control sides (Tab 1).

Radiographic Evaluation and Mechanical Testing

In the 14 days consolidation experiment, X-rays showed that the radiodensity at the treatment side was greater than the control side in most cases (6/8, Fig. 2), and the maximum load in the treatment side was significantly higher than in the control side (p<0.01, Fig. 3). Similarly, hNGFβ increased the maximum load by 23% in the 28 days consolidation experiment (p<0.01, Fig. 3).

| Table 1. Callus dimensions and histomorphometric analysisa |
|------------------------|------------------------|------------------------|------------------------|
|                        | 14 days of consolidation | 28 days of consolidation |
|                        | Control | Treatment | Control | Treatment |
| Distance achieved (mm) | 9.42±0.21 | 9.35±0.25 | 9.33±0.14 | 9.40±0.32 |
| Labial-lingual dimension (mm) | 6.99±0.44 | 6.92±0.91 | 6.82±0.35 | 6.79±0.27 |
| Superior-inferior dimension (mm) | 12.72±0.78 | 12.59±1.05 | 12.62±1.14 | 12.39±0.82 |
| Bone volume/total volume (%) | 33.0±4.9 | 49.8±11.6* | 52.8±8.8 | 66.5±14.9* |
| Number of osteoblasts (/mm²) | 208.3±43.3 | 214.8±36.5 | 274.5±39.8 | 270.9±58.7 |
| Mineralized bone percentage (%) | 30.7±4.5 | 42.1±8.6* | 49.1±9.0 | 58.4±12.8* |

* Mean and standard error of the mean.
* p<0.01 compared with the control side after the same days of consolidation.

Bone Histology and Histomorphometry

In the 14 days consolidation experiment, the distraction gaps of both treatment and control sides were completely united mainly with woven bone and there was initial replacement of woven bone by lamellar bone. The bony trabeculae rimmed by osteoblasts showed orientation along the axis of mechanical force. In comparison, the bony trabeculae were thicker in the treatment side (Fig. 4). In the 28 days consolidation experiment, the bony trabeculae became thicker in both sides, also rimmed by osteoblasts. The structure of bony trabeculae was a mixture of woven and lamellar in the control side and mostly lamellar bone in the treatment side. hNGFβ increased BV/TV by 51% (p<0.01) and 26% (p<0.01) in the 14 and 28 days consolidation experiments respectively (Tab 1). Similarly, hNGFβ increased mineralized bone percentage by 37% (p<0.01) and 19% (p<0.01) in the two consolidation experiments respectively (Tab 1). The number of osteoblasts/mm² was not significantly different between the treatment and control sides (Tab 1). In both 14 and 28 days consolidation experiments, the mineral apposition rate of the 1st-11th day after distraction was significantly higher in the treatment side compared with the control side (p<0.01), but the rate of the 12th-22nd day after distraction was not significantly different between the treatment and control sides in the 28 days consolidation experiment (Tab 2).
DISCUSSION

Many attempts have been made to shorten the bone consolidation period during DO, including low-intensity pulsed ultrasound, recombinant growth hormone, intermittent parathyroid hormone, calcitonin and alendronate, calcium sulphate, and growth factors like BMPs and IGF. In the present study, locally applied hNGF accelerated callus maturation of a distraction gap in a rabbit mandibular distraction model.

Distraction models in the mandible have been following the way of successful distraction models in long bone for many years. But there are some differences between the mandible and a long bone such as tibia in rabbits. The mandible is mainly composed of cancellous bone with much thinner cortex than in tibia. Therefore, the callus formed in the mandible distraction is more uniform in cancellous bone and cortex than in a long bone distraction and easier for histological evaluation. Besides, the more abundant blood supply in the mandible makes a shorter latency period possible. In this distraction model, we applied a distractor suitable for simultaneous bilateral distraction of the body of rabbit mandibles without the need for two separate devices. Another advantage of this model is the establishment of an intra-individual placebo control. The ‘clap-shaped’ distraction device in this model may also be adapted to a future model of simultaneous distraction in parallel long bones, such as radius/ulna and tibia/fibula. The possible advantages include providing a precise simultaneous distraction of both long bones and the smaller size which causes less inconveniences.

When starting to use this model in our previous studies, despite injection of antibiotics, we observed many local infections in rabbits of which subcutaneous abscess is a major type (unpublished data). The high infection rate in these studies could be mainly attributed to a less meticulous surgery technique and contamination of the midline incision during eating or drinking. The incidence of local infection in the present study has been reduced to 10.5% (2/19) by improving the surgical techniques and applying an antimicrobial ointment to the wound.

The rabbit models of leg or mandible lengthening are well-established and have been used extensively to perform callus stimulation studies. Previous reports using these models have shown that the optimal rate of lengthening is about 1.0 mm daily; faster rates (>1.3 mm/day) can result in a poor quality of bone formation.

**Fig 3.** The effect of hNGFβ on the maximum load in a three-point bending test. *p < 0.01 compared with the control side after the same days of consolidation.

**Fig 4.** The effect of hNGFβ on the histology of the distracted callus in the 14 days consolidation experiment. (A) treatment side, (B) control side (Hematoxylin and eosin staining, bar length 0.5 mm).
Although fast rates (1.5 or 2 mm/day) were deliberately chosen to establish models of poor bone formation in many previous calss stimulation studies, a slow rate of 0.5 mm per 12 hours was chosen in the present study to mimic normal clinical situations of distraction osteogenesis. We believe that this slow rate with the application of the NGF along with the latency period can be of more important clinical significance. Based on the understanding of a quicker healing process in rabbits than in humans, different latency periods such as 5 days and 3 days were recommended in previous studies on rabbit models of mandibular distraction osteogenesis. If the latency period is too short, released soft tissue and periostemeum can not gain their recovery and there are not enough osteogenitor cells ready for the strain. But if the latency period is too long, premature consolidation may occur and distraction is consequently difficult. In the present model, we observed that an average of 2.5 days was needed for rabbits to adapt to the distraction device and regain full masticatory. Thus, we assumed that soft tissue and periostemeum could gain their recovery after 3.5 days and a 3.5 day latency period was suitable.

In DO, it has been demonstrated that strain is the key stimulator of the osteogenic cells, in which expression of growth factors and signaling factors is increased as long as the distraction is maintained and quickly downregulated as soon as the distraction completes. Therefore, the beginning of consolidation period are very likely to be a key period needing exogenous growth factors. Based on the findings of BMPs expression profile, single or repeated injections of BMPs applied at the beginning of consolidation period resulted in enhanced bone consolidation in animal models of DO. Because the expression pattern of NGF is very likely to mimic the expression profile of BMPs during distraction osteogenesis, NGF injections were carried out at the beginning of consolidation period (about 2 weeks after osteotomy) in the present study. In order to reduce the error of injection procedure, we chose repeated injections instead of a single injection. Besides, an interval period of 2 days was also designed to allow the rabbits to recover from injury of puncture. Accelerated callus maturation of the distraction gap demonstrates that the beginning of consolidation period is very likely to be an important period needing exogenous NGF.

In this study, mechanical testing showed that the maximum load on the treatment side was significantly higher than that on the control side in both 14 and 28 days consolidation experiments. Because no difference on the callus dimensions was observed between the treatment and control sides, the major reason for the increased maximum load should be the improved arrangement and dimension of each trabecula and better bone mineralization. Further, bone histological and histomorphometric analysis demonstrated that hNGFβ improved the density and quality of regenerate trabecula. Interestingly, no significant difference was detected on the number of osteoblasts between the treatment and control sides. This can be partly explained by the previous findings that NGF can enhance differentiation of the existing population of osteoblasts without enhancing their proliferation. Besides, we detected a significant difference in mineral apposition rates between the treatment and control sides. Such a difference reflects the effect of hNGFβ on individual osteoblasts.

Our study has limitations, and a variety of problems await further studies. First, we chose the dose of 40 µg hNGFβ only on data of a preliminary study, not on that of release kinetics or clearance rate. Second, we did not follow the whole healing process until the end of optimal consolidation; and did not test the influence of different distraction speeds, distances and doses of hNGFβ on callus maturation. Third, the use of carrier materials is very important to protect the locally applied soluble protein factors from inactivation. Although the distraction tissue that formed after 10 days of distraction may be considered as a matrix for the injected hNGFβ and a clear effect on callus maturation was observed, the administration need to be improved, for example by applying injectable or degradable carrier materials. Fourth, the potential ability of promoting the recovery of inferior alveolar nerve injury, one of the most important characteristics of NGF, has not been evaluated in this study. Moreover, many issues such as proper dose and adequate frequency of injections remain unanswered for future clinical application of hNGFβ.

Nonetheless, we have demonstrated that locally applied hNGFβ can accelerate callus maturation in a rabbit model of mandibular distraction osteogenesis. It may therefore play a role in shortening the consolidation period and lowering

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<th>Table 2 Mineral apposition rate analysesa</th>
<th>14 days of consolidation</th>
<th>28 days of consolidation</th>
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<tr>
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<td>Day 1-11</td>
<td>Day 11-11 Day 12-22</td>
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<tr>
<td>Control</td>
<td>2.41±0.27</td>
<td>2.43±0.21 1.71±0.16</td>
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<tr>
<td>Treatment</td>
<td>3.20±0.69a</td>
<td>3.13±0.36 1.79±0.38</td>
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* Mean and standard error of the mean. (µm/day)
* p<0.01 compared with the control side after the same days of consolidation.
the number of complications in mandibular or long bone DO clinically.

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