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Immunohistochemical study of vascular endothelial growth factor (VEGF) and bone morphogenetic protein-2, -4 (BMP-2, -4) on lengthened rat femurs

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SUMMARY. Background: **With a hypothesis that "angiogenesis occurs before osteogenesis," an experimental study using a rat model was carried out. Histological and immunohistochemical examinations of vascular endothelial growth factor (VEGF) and bone morphogenetic protein-2, -4 (BMP-2, -4) were performed at the margins of bone formation after femoral bone lengthening.** Material and methods: **Thirty-five Wistar rats weighing 380–400 g (11-week-old males) were used. An external fixator was applied on the femur, and an osteotomy performed under general anaesthesia. Five days after the operation, femoral lengthening was initiated at a rate of 0.8 mm/day for 8 days. The rats were sacrificed just after distraction was completed, and at 1, 3, 5, 7, 9 and 14 days after distraction. The specimens from these rats were stained with haematoxylin–eosin, VEGF, and BMP-2, -4 immunohistochemical staining, and were investigated.** Results: **Expression of VEGF was observed in the woven bone at the osteogenetic front and near to osteoblasts around the newly formed bone. On the other hand, expressions of BMP-2, -4 were seen in the hypertrophic chondrocytes. In the same specimen, the VEGF area was further away from the bone stump than the BMP-2, -4 areas.** Conclusion: **These results confirm the hypothesis that angiogenesis is induced before osteogenesis.** © **2005 European Association for Cranio-Maxillofacial Surgery**

Keywords: **distraction osteogenesis; bone formation; vascular endothelial growth factor (VEGF); bone morphogenetic protein (BMP)**

INTRODUCTION

Since *McCarthy* et al. (1992) described distraction osteogenesis in the oral and maxillofacial skeleton, the technique has been widely used and accepted as an option in patients with micrognathia or other bone defects. However, it requires a considerable time to lengthen bone. The process consists of three stages; latency, lengthening and consolidation.

Many attempts have been made to promote bone formation and to shorten the treatment period using electrical stimulation (*Hagiwara* and *Bell*, 2000), low-intensity pulsed ultrasound stimulation (*Shimazaki* et al., 2000), and oxygen exposure (*Kitakoji* et al., 1999). It has been reported that an injection of cells or cytokines into the distracted callus enhanced the bone formation and shortened the consolidation period. It is important to know the best timing for stimulation, and what kind of stimulation is the best to enhance "histogenesis". With an hypothesis that "angiogenesis occurs before osteogenesis," the front of bone formation after femoral lengthening in rats was investigated by immunohistochemical staining of vascular endothelial growth factor (VEGF) and bone morphogenetic protein-2, -4 (BMP-2, -4).

MATERIAL AND METHODS

Animals

Thirty-five Wistar rats weighing 380–400 g (11-week-old males) were used. The protocol and guidelines for this study were approved by the Institutional Animal Care and Use Review Committee of the Nagoya University School of Medicine, Nagoya, Japan. The animals were procured from the Nagoya University Experimental Animal Centre. The preoperative and postoperative care of these animals was overseen by university veterinary technicians to ensure proper treatment.

Surgical procedure

Before surgery, the animals were anaesthetized using pentobarbital sodium (30–40 mg/kg) by intraperitoneal injection. Approximately 0.9 ml of local anaesthesia (2% lidocaine with epinephrine 0.01 mg/ml) was injected into the surgical sites. A lateral longitudinal skin incision was made over the left femur, the fascia was incised and the quadriceps femoris and hamstrings were separated. After predrilling with a

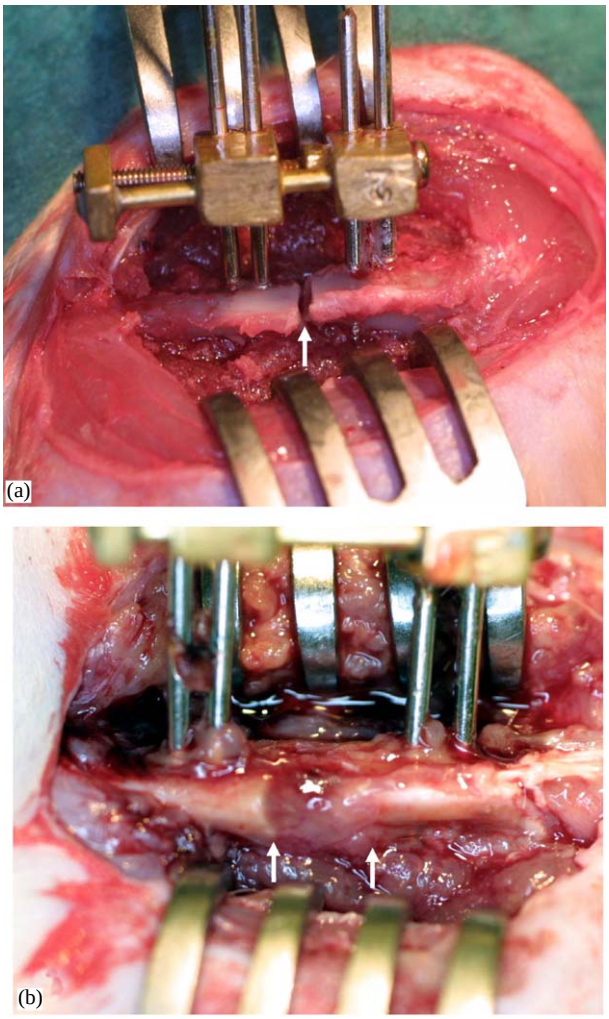

Fig. 1 – Distraction device and lengthened bone. (a) Complete osteotomy (arrow). (b) Lengthened bone 5 days after distraction. Distracted callus was seen (arrows).

fissure bur, a hand-made external fixator was fixed with four Kirschner-wires (1.4 mm in diameter). Osteotomy was performed between the second and third Kirschner wires using a fissure bur under irrigation with physiological saline (Fig. 1). The fascia and the skin were sutured using absorbable surgical sutures. The rats were allowed free movement in their cages after anaesthesia.

Experimental protocol

Five days after operation, femoral lengthening was initiated at a rate of 0.8 mm/day for 8 days. The rats were divided into 7 groups and were sacrificed just after distraction, at 1, 3, 5, 7, 9 and 14 days after distraction. The left femur was resected with surrounding tissues (Fig. 2). The specimens from these rats were evaluated using radiological, histological and immunohistochemical analysis.

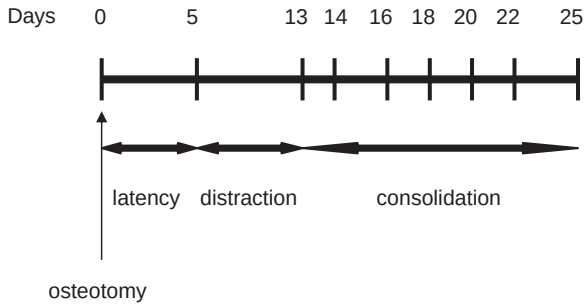

Fig. 2 – Schematic representation of the study protocol. Time points indicate postoperative days.

Radiographic evaluation

Radiographs of the distracted femurs were taken with a soft X-ray apparatus (Softex CSM-2, Softex Co., Tokyo, Japan; 70 kV, 3 mA).

Histological and immunohistochemical assessment
Specimens for histological and immunohistochemical staining were fixed with periodate-lysin-paraformaldehyde (PLP) fixative (ph 7.4) at 4 °C for 48 h. After being washed in phosphate-buffered saline (PBS), they were demineralized with ethylenediaminetetraacetic acid (EDTA)-glycerol solution (ph 7.4) for 4 weeks. They were washed in PBS, dehydrated in graded concentrations of ethanol, embedded in paraffin, and cut into sections of 4 µm thickness through the long axis. Serial sections were mounted on slides, and dried overnight. Sections were stained with haematoxylin–eosin, immunohistochemical staining for VEGF and BMP-2, -4.

VEGF
All steps were carried out at room temperature in a humidified chamber. After deparaffinization and hydration, endogenous peroxidase was blocked with 1% hydrogen peroxide for 5 min. After blocking with 1% goat serum in PBS for 30 min, the sections were incubated with commercially available monoclonal mouse anti-VEGF antibodies (SantaCruz Biotechnologies, Inc., USA) for 2 h. For negative controls, the primary antibody was omitted. The sections were rinsed with PBS and were subsequently reacted with biotinylated goat anti-mouse IgG for 30 min. They were incubated in 3, 3-diaminobenzidine tetrachloride solution (DAB). Finally, the sections were counterstained with haematoxylin and mounted.

BMP-2, BMP-4
After deparaffinization and hydration, endogenous peroxidase was blocked with 1% hydrogen peroxide for 10 min. After blocking with 1% rabbit serum in PBS for 30 min, the sections were incubated with commercially available polyclonal goat anti-BMP-2, -4 antibodies (SantaCruz Biotechnologies, Inc., USA) at 4 °C overnight. For negative controls, the primary