Expression of Bone Morphogenetic Proteins during Mandibular Distraction Osteogenesis

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Distraction osteogenesis is a form of in vivo tissue engineering in which the gradual separation of cut bone edges results in the generation of new bone. In this study, the temporal and spatial expression of bone morphogenetic proteins (BMPs) 2, 4, and 7 was examined in a rabbit model of mandibular distraction osteogenesis. Fourteen skeletally mature male rabbits were studied. After osteotomy, a distractor was applied to one side of the mandible. After 1 week of latency, distraction was initiated at 0.25 mm every 12 hours for 3 weeks (distraction period), followed by a 3-week consolidation period. Two animals were killed each week after surgery. The regenerate bone was analyzed for the expression of BMP-2, -4, and -7 by using standard bone histological and immunohistochemical techniques. BMP-2 and -4 were highly expressed in osteoblastic cells during the distraction period and in chondrocytes during the consolidation period. BMP-7 demonstrated relatively minor expression in osteoblastic cells during the distraction period. All BMPs were strongly expressed in vascularized connective tissue during the distraction period. These data indicate that BMPs participate in the translation of mechanical stimuli into a biological response during mandibular distraction osteogenesis. (Plast. Reconstr. Surg. 111: 201, 2003.)

Distraction osteogenesis is a form of in vivo tissue engineering in which the gradual separation of cut bone ends results in the generation of new bone. The biomechanical principles governing distraction osteogenesis were first studied by Ilizarov\textsuperscript{1,2} in the early 1950s. The mechanisms by which the mechanical stimulus is translated into biological signals remain poorly understood, however.

Bone morphogenetic proteins (BMPs) are ubiquitous multifunctional proteins that have widespread effects on cell growth and differentiation in many organ systems.\textsuperscript{3,4} Seventeen BMPs have been characterized and cloned to date, and all of them except BMP-1 are members of the transforming growth factor-\(\beta\) superfamily.\textsuperscript{4} Among these, BMP-2, -4, and -7 demonstrate the greatest osteogenic activity.\textsuperscript{4} Recombinant BMP-2, -4, and -7 are able to induce ectopic bone formation, eliminating the need for other growth factors in this process.\textsuperscript{5,6}

In accordance with the known importance of BMPs in osteogenesis, these molecules have been demonstrated to be involved in distraction osteogenesis of long bones.\textsuperscript{7–9} In a rabbit tibia model of distraction osteogenesis, BMP-4 was expressed by less-well differentiated osteoprogenitor cells but not by fully differentiated osteoblasts.\textsuperscript{7} In a rat femur model, expression of BMP-2 and -4 was markedly increased in chondrogenic precursor cells during the early stages of distraction, whereas the signals for BMP-6 and -7 were not enhanced.\textsuperscript{8} Our own rabbit tibia studies demonstrated high levels of expression of BMP-2, -4, and -7 during the distraction phase, with undetectable signals during the consolidation phase.\textsuperscript{9}

The role of BMPs in mandibular distraction osteogenesis has not been previously examined. It has been demonstrated, however, that BMPs are expressed during the healing of mandibular fractures.\textsuperscript{10,11} Expression of BMP-2 mRNA was reported to be greatest at the stage of intramembranous bone formation and early chondrogenesis.\textsuperscript{10} BMP-2, -4, and -7 proteins

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were expressed by osteoblasts, osteoclasts, and more primitive mesenchymal cells within the fracture callus during the early stages of membranous fracture healing.\textsuperscript{11}

In this study, we characterized the protein expression of BMP-2, -4, and -7 during distraction osteogenesis of the rabbit mandible. The methods used were the same as in our previous studies on distraction osteogenesis in rabbit tibiae, allowing comparison of the findings at the two skeletal sites.

**MATERIALS AND METHODS**

**Animals**

Fourteen skeletally mature (9-month-old), male, white New Zealand rabbits weighing 3.5 to 4.5 kg were studied. The housing, care, and experimental protocol were approved by the McGill University Animal Care and Ethics Committee.

**Operative Technique**

A longitudinal skin incision was made along the inferior border of the left mandible. The subcutaneous tissues were divided with a handheld cautery. The platysma was transected and reflected in a plane above the periosteum. The inferior alveolar nerve and the anterior aspect of the first premolar tooth were then identified. A Hall drill with a 1.5-mm drill bit was used to drill through the lateral and medial cortices of the mandible in two standard locations. The first hole was drilled just inferior to the inferior alveolar nerve. The second hole was drilled 1.4 cm posterior to the first hole. Two self-tapping, 2-mm, titanium screws were then inserted into the holes, as shown in Figure 1.

A minimum of periosteum was incised longitudinally along the inferior border of the left mandible and was carefully elevated. A transverse osteotomy was made with an oscillating saw, just anterior to the first premolar. The screws were fixed to a modified Orthofix uniplanar M-100 fixator (Orthofix Inc., Verona, Italy), and the cut edges of bone were reapproximated. The wound was closed in two layers, using 3-0 Vicryl for the platysma and 3-0 silk for the skin. This procedure was performed on the left side of the mandible only; the right side served as a control.

**Experimental Protocol**

The experimental protocol used was identical to that previously described for tibial distraction osteogenesis\textsuperscript{9} (Fig. 2). After surgery, the animals were allowed to heal for 1 week (latency period). After this delay, distraction was initiated at a rate of 0.25 mm every 12 hours for 3 weeks (distraction period). This was followed by a 3-week period during which the external fixator was held in place without distraction (consolidation period).

Each week after surgery, two rabbits were killed with an intravenous injection of pentobarbital (100 mg/kg), and the mandible was resected en bloc. A plain, superior-view radiograph of the mandible was obtained. The surrounding soft tissues were then carefully removed, and the generate bone was harvested for histological and immunohistochemical assessments. The non-surgically treated contralateral side of each mandible was also harvested, for control histological and immunohistochemical assessments.

**Histological and Immunohistochemical Assessments**

After harvesting, the generate bone and the control specimen were fixed in 10 percent neutral buffered formalin and embedded in methacrylate. Sections were cut at low speed, using a motorized microtome (Polycut E; Reichert-Jung, Heidelberg, Germany), and placed on gelatin-coated slides. For histological evaluations, tissues were stained with Goldner trichrome stain.

Immunohistochemical assays were performed basically as previously described.\textsuperscript{9} To optimize staining in mandibular bone, the sections were deacrylated in 2-methoxyethyl ace-
tate. Endogenous peroxidase was inhibited with 3 percent hydrogen peroxide and blocked with 10 percent normal horse serum before staining. Commercially available, polyclonal, goat anti-BMP-2, -4, and -7 antibodies were used for immunostaining (Santa Cruz Biotechnology, Santa Cruz, Calif.). Sections were incubated with these primary antibodies overnight at 4°C, in a humidified chamber. A biotinylated horse anti-goat immunoglobulin antibody was used as a secondary antibody (Vector Laboratories, Burlingame, Calif.). The Vectastain-Elite avidin-biotin complex detection system, diaminobenzidine revelation kit (Vector), and diaminobenzidine enhancing solution (Vector) were then used to complete the immunostaining. Finally, the sections were counterstained with Mayer’s hematoxylin and mounted.

According to data provided by the manufacturer, the primary antibodies used in this study are known to recognize mouse, rat, and human BMPs. Therefore, we tested whether these antibodies also recognize rabbit BMPs, to confirm that the observed staining patterns represented BMP-specific signals. Following the instructions provided by the manufacturer, 100 μl of goat BMP blocking peptide (concentration, 200 μg/ml) was centrifuged in a SpeedVac concentrator (Savant, Farmingdale, N.Y.), yielding the blocking peptide in a powder form. This was mixed with 20 μl of primary antibody (concentration, 200 μg/ml) and preincubated overnight at 4°C. Subsequently, the protocol used was the same as for the sections without blocking peptides. When sections were treated as described above, no staining was evident. Therefore, the antibodies used in this study recognized rabbit BMPs.

The numbers of cells expressing BMP-2, -4, and -7 were assessed by using standard cell-counting techniques. Chondrocytes, osteoblastic cells, and fibroblastic cells were identified morphologically and assessed for BMP expression. Osteoblasts were defined as cells that were directly apposed to bone surfaces and exhibited a definite Golgi apparatus. Fibroblasts were recognized as cells within the stroma that were of elliptical shape. Cells were regarded as chondrocytes when they were surrounded by cartilaginous matrix and exhibited a nearly circular shape. Cell counting was performed for all tissue found in the distraction zone.

RESULTS

Clinical Outcomes

All 14 animals tolerated the surgery/distraction protocol. None of the animals experienced postoperative complications, such as aspiration pneumonia or wound infections. The animals were fed regular chow, and all gained weight. By the second week of distraction, the rabbits developed a significant crossbite and overgrowth of their incisors. Two animals required trimming of the incisors for comfort and facilitation of feeding.

Radiographic Appearance

Immediately after killing, the mandible and overlying soft tissues were resected en bloc and plain x-ray films were obtained from a superior view (Fig. 3). After the latency period (week 1
group), radiographs revealed good reduction of the osteotomy and proper alignment of the proximal and distal mandibular segments. The fracture line was also apparent, albeit subtly. These findings suggest that the distraction device was properly applied and provided adequate rigid external fixation throughout the latency period, without abnormal torque.

**Histological Results**

Goldner trichrome staining revealed that, before distraction (week 1), fibrous tissue and hemorrhage were observed in the osteotomy region (data not shown). By the end of the first week of distraction (week 2), new longitudinal bone trabeculae had formed on both sides of the distraction gap (Fig. 4). Significant amounts of highly vascularized connective tissue, osteoblastic and fibroblastic cells, and cartilaginous areas were identified throughout the distraction period (weeks 2 to 4), as presented in Figure 4.

After 2 weeks of consolidation (week 6), the distraction gap was bridged by trabeculae consisting of woven bone (Fig. 4). A few islands of cartilage could also be observed. By week 7, the distraction gap was essentially ossified, and the osteotomy edges were poorly demarcated.

**Immunohistochemical Results**

The results of qualitative evaluation of BMP expression are presented in Table I. Representative examples of immunostained sections from week 2 to week 7 are illustrated in Figure 5. Intense staining for BMP-2, -4, and -7 was evident in osteoblastic cells 1 week after surgery, even before distraction was initiated (Table I). There was no positive signal in the control mandible.

A significant amount of staining for BMP-2,
-4, and -7 was also noted within the vascularized connective tissue throughout the distraction period (Table I). Moderate signals for BMP-2, -4, and -7 were detected in osteoblastic cells during the first week of distraction. Moderate signals for BMP-2 and -4 only were detected in chondrocytes during the last week of distraction (week 4).

During the consolidation period (weeks 5 to 7), BMP-2 and -4 were strongly expressed in chondrocytes, as shown in Table I and Figure 5. BMP-7 was minimally expressed during the consolidation period.

DISCUSSION

In this study, we present histological and immunohistochemical analyses of distraction osteogenesis in the rabbit mandible. Our his-
tological findings are similar to observations reported by others. Bone was formed primarily through intramembranous ossification during the distraction period, but endochondral ossification was also present during the consolidation period. The presence of chondrocytes in the mandibular distraction zone regenerate may indicate a lack of stability of the distraction device (a possibility with a two-pin device). These tissue-level features of mandibular distraction osteogenesis are similar to those of long-bone distraction osteogenesis.

Our immunohistochemical studies demonstrated that BMPs were strongly expressed in osteoblastic cells even before distraction was initiated. This finding is consistent with reports of BMP mRNA expression during fracture healing in the rabbit mandible. In 1997, Si et al. used an in situ hybridization technique to demonstrate BMP-2 mRNA expression in undifferentiated mesenchymal cells following mandibular fracture in rabbits. The BMP-2 signal was greatest at the stage of intramembranous bone formation and early chondrogenesis, suggesting that BMP-2 mediates the differentiation of mesenchymal cells into osteoblasts and chondroblasts. More recently, Spector et al. studied the expression of BMP-2, -4, and -7 during fracture healing in the rat mandible, using immunohistochemical analyses. All three BMPs were expressed by osteoblasts, osteoclasts, and more primitive mesenchymal cells within the fracture callus during the early stages of membranous fracture healing.

During the first 2 weeks of distraction, very intense signals for BMP-2, -4, and -7 were detected in vascularized connective tissue. Subsequently, these signals decreased considerably or were absent during the consolidation period. The amounts of vascularized connective tissue in the distraction gap also exhibited this temporal pattern. Our findings are thus consistent with the hypothesis that BMPs play a role in the angiogenic response to the distraction stimulus.

This study demonstrates that the general pattern of BMP expression during mandibular distraction osteogenesis resembles our earlier observations on distraction osteogenesis of the tibia. At both skeletal sites, BMP expression levels were highest during distraction and decreased during the consolidation phase. Nevertheless, some differences between the two sites were observed (Fig. 6). In particular, BMP-7 exhibited intense staining in fibroblasts and chondrocytes during tibial distraction but was minimally expressed in those cell types during mandibular distraction osteogenesis. Also, BMP-2 and -4 were expressed in chondrocytes during the consolidation period in the mandible, in areas corresponding to focal ar-

| Table I |
| Qualitative Analysis of Bone Morphogenetic Protein Expression during Distraction Osteogenesis of the Mandible |

| Protein | Week | Osteoblastic Cells (Preosteoblasts) Chondrocytes Fibroblastic Cells Vascularized Connective Tissue |
|---------|------|---------------------------------|---------------------------------|---------------------------------|
| BMP-2   | 1    | +++                           | -                              | +++                            |
|         | 2    | ++                            | +                              | +                              |
|         | 3    | +                             | +/-                            | -                              |
|         | 4    | -                             | +++                            | +                              |
|         | 5    | -                             | +++                            | +                              |
|         | 6    | -                             | +++                            | -                              |
|         | 7    | -                             | -                              | -                              |
| BMP-4   | 1    | +++                           | -                              | +++                            |
|         | 2    | ++                            | +                              | +                              |
|         | 3    | +                             | +/-                            | -                              |
|         | 4    | -                             | +++                            | +                              |
|         | 5    | -                             | +++                            | +                              |
|         | 6    | -                             | +++                            | -                              |
|         | 7    | -                             | -                              | -                              |
| BMP-7   | 1    | ++                            | -                              | +++                            |
|         | 2    | +                             | +/-                            | -                              |
|         | 3    | +                             | -                              | -                              |
|         | 4    | -                             | ++                             | +                              |
|         | 5    | -                             | +                              | -                              |
|         | 6    | -                             | +                              | -                              |
|         | 7    | -                             | -                              | -                              |

- , no positive staining; +, less than one-third of cells positive; ++, one-third to two-thirds of cells positive; ++++, more than two-thirds of cells positive; week 1, latency period; week 2 to 4, distraction period; week 5 to 7, consolidation period.
eas of endochondral ossification. This was not observed in tibial distraction osteogenesis. The differences between mandibular and tibial distraction osteogenesis might be attributable to the different ontogenic origins (membranous versus endochondral) of the bones or might reflect differences in the mechanical environments at the two sites.

In conclusion, these data indicate that the changes in the mechanical environment created by distraction lead to increased BMP expression during mandibular distraction osteogenesis. From a more clinical perspective, our observations suggest that future attempts to accelerate the distraction process with exogenous BMP administration should focus on the consolidation period, when endogenous BMP production declines in the bone-forming osteoblasts.

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