Effects of osteogenic protein-1 on distraction osteogenesis in rabbits

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Abstract

In this study we tested the effect of locally applied osteogenic protein 1 (OP-1) on distraction osteogenesis in rabbits. Seven days after tibial osteotomy, distraction was started at a rate of 0.25 mm per 12 h for 3 weeks. At the end of the distraction period, OP-1 was injected at the site of osteotomy. Four different dosages were tested (0, 80, 800, or 2000 μg; eight rabbits per dose group). Rabbits were sacrificed 3 weeks later, and histologic, densitometric, and biomechanical parameters were assessed. No significant differences were found between groups for any parameter. To explain why this approach was only modestly successful, the expression of BMP receptor protein in the newly formed tissue was analyzed by immunohistochemistry. Strong expression of BMP receptor IA, IB, and II was found during the early distraction phase, but not during later stages of the process. Thus, it appears that the lack of receptor protein in the target tissue impairs the effect of OP-1 given at the end of the distraction period. Possibly, OP-1 could be more useful when applied early in the distraction phase.

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Introduction

Distraction osteogenesis (DO) is a well-established technique for bone lengthening that has widespread clinical applications in the treatment of limb length discrepancies, bone defects, limb deformities, and fracture nonunion [1,2]. An osteotomy is performed, followed by fixation with an external fixator. After a latency period of about a week, the osteotomy is subjected to controlled distraction. Thereby, osteogenesis is induced and the bone continues to grow in length as long as the distraction is maintained at an adequate rate and rhythm. When distraction is stopped, bone lengthening ceases and the newly formed bone in the distracted zone gradually consolidates.

Although DO has revolutionized the treatment of many orthopaedic disorders, one of the problems of this technique is the long period during which external fixation is required until the newly formed bone consolidates (approximately 1–2 months for every centimeter lengthened). This can cause significant morbidity to the patient [3]. Various approaches have been tested to accelerate osteogenesis in this setting, such as mechanical compression [4,5], electrical and electromagnetic stimulation [6,7], low velocity ultrasound [8], growth hormone [9], Prostaglandin E2 [10], bone marrow extracts [11,12], demineralized bone matrix [13], osteoblast-like cells [14,15], and certain growth factors including TGFβ [16,17] and fibroblast growth factor [18]. Some of these studies have yielded promising results, but as of yet, none of these approaches have been shown to be efficient in humans.

Bone morphogenetic proteins (BMPs) are a group of growth factors that play an important role in bone formation. BMPs are potent inducers of osteogenesis both during embryological bone formation and in fracture repair [19–21]. Recombinant BMP-7 (also called osteogenic protein 1 or OP-1) has been shown to accelerate the formation of new bone in numerous preclinical and clinical studies [22–24], including healing of critical-sized defects of long bones.
[25–28], healing of diaphyseal nonunions [29], enhancement of bone grafts and bone substitutes [30–32], and substitutes for bone grafts in spinal fusions [33,34].

We have previously shown that the expression of BMP-2, 4, and 7 proteins was maximal during the distraction phase of DO, but that BMP expression tapered off during the consolidation phase [35]. We therefore hypothesized that the best time for local application of exogenous BMPs would be at the start of the consolidation phase. The first aim of this present study was to investigate whether a single injection of OP-1 applied locally at the distracted zone at the beginning of the consolidation phase, could accelerate the consolidation of regenerate bone.

Most BMPs exert their biological effects by binding to one of two kinds of BMP receptors (BMPR), designated type I and type II. Two different type I BMPR (IA and IB) and one type II BMPR have been identified [36,37]. While the expression of these receptors during embryological bone formation and fracture repair has been extensively investigated [38,39], their expression in DO remains largely unknown. Therefore, the second aim of this study was to investigate the immunolocalization of BMP receptors in DO.

### Material and methods

#### Operative protocol

A total of 50 skeletally mature (9-months old) male white New Zealand rabbits weighing 3.5 to 4.5 kg were studied. The operative and distraction protocol were identical to our earlier studies [16,35]. The rabbits were anesthetized by intramuscular administration of ketamine (30 mg/Kg) and xylazine (5 mg/kg). After intubation, anesthesia was maintained with halothane, oxygen, and nitrous oxide after intubation. A modified Orthofix Uniplanar M-100 fixator (Orthofix, Inc., Verona, Italy) was applied to the left tibia under sterile conditions. Four half-pins were inserted, two above and two below the osteotomy site. The tibia was exposed subperiosteally and an osteotomy was performed with an oscillating saw just below the fusion site of the tibia and fibula. The periosteum was reapproximated and the wound closed. Unrestricted weight bearing and activity were allowed postoperatively.

After a latency period of 7 days, distraction was started at a rate of 0.5 mm per 12 h for 3 weeks (distraction phase). This was followed by a period of 3 weeks, during which the external fixator was held in place without any distraction (consolidation phase). All rabbits assigned for bone densitometry, biomechanical testing, and histomorphometry were sacrificed 7 weeks after the surgery. As to the six rabbits assigned for immunohistochemistry, one rabbit was sacrificed at each week starting 1 week after surgery. The McGill University Animal Care and Ethics Committee approved the housing, care, and experimental protocol. All the animals were sacrificed by intravenous euthanyl (sodium pentobarbital) 1 ml/kg.

#### Animal groups

As shown in Table 1, the rabbits were randomly divided into five groups; in Group I, only simple lengthening without any injections was performed. In Group II, acetate buffer (carrier) was injected at the end of distraction. Groups III, IV, and V, received injections of 80, 800, and 2000 μg of OP-1, respectively. Under X-ray control, a single bolus injection was applied to the center of the regenerate zone at day 21 of distraction (Fig. 1). Details of the length, width, and volume of the regenerate zone at that

![Fig. 1. (a) External fixator used for DO in the rabbits. (b) X-rays at the end of distraction showing needle at the middle of the distracted zone during injection.](image_url)
time are shown in Table 2. A 23-gauge needle was used for a total volume of 0.2 ml. The OP-1 (kindly donated by Stryker Biotech. Inc, Hopkinton, MA, USA) was stored at 20°C until use.

After sacrifice, the hip joint was disarticulated and both lower limbs were wrapped in moist saline dressing. Samples assigned for immunohistochemistry were immediately processed (see below). The other specimen underwent bone densitometry. These samples were then either placed in formalin for histomorphometry processing or stored frozen for biomechanical testing.

**Bone densitometry**

Dual X-ray absorptiometry was performed using a Hologic QDR 2000W device (Hologic Inc., Waltham, MA, USA). Bone mineral content (BMC) and areal bone mineral density (BMD) were determined in the lengthened zone of the right tibiae and at a corresponding location of the non-operated left tibia, as described [16].

**Biomechanical testing**

The specimen were stored at −20°C until the day of mechanical testing. Upon thawing, the tibiae were cleaned of all excess soft tissue. The length of the tibiae as well as the maximal anteroposterior and lateral diameter of the callus were measured using a digital caliper. Both metaphyseal ends of each specimen were embedded in polymethyl metacrylate (PMMA) fixation blocks (diameter 45 mm) to a depth of approximately 25 mm. The diaphyseal area, including the distraction region and both adjacent pin holes, were free and centered between the two PMMA blocks.

The PMMA blocks were placed within holding cups and then mounted to the two platforms of the testing machine (856 Mini Bionix, MTS, Eden Prairie, MN USA) with an universal joint. Starting at zero load, the actuator was moved with a constant speed of 0.1 mm per second upward, loading the tibia in axial tension until mechanical failure. Axial loads were recorded throughout a 50 Hz sampling rate. From the load displacement curves, the ultimate tensile load, stiffness, and energy-to-failure were calculated. Biomechanical testing was also performed on all the contralateral nonoperated tibiae.

**Histomorphometry**

Samples assigned for histomorphometry were embedded undecalcified in PMMA. Sections of 6-μm thickness were stained with toluidine blue and Goldner trichrome. Sections were analyzed with a Polyvar microscope (Reichert-Jung, Heidelberg, Germany) at an 80-fold magnification. Quantitation was performed using a digitizing tablet and the Os- teomeasure® software (Osteometrics, Atlanta, GA, USA). Nomenclature follows the recommendations of the American Society for Bone and Mineral Research [40]. The defined region of interest was the entire distraction gap (Fig. 2). The number of chondrocytes, osteoblasts, fibroblasts, and fibroblast-like cells was calculated as a percentage of the total volume, the remainder representing bone marrow.

**Immunohistochemistry**

In samples assigned for immunohistochemistry, soft tissues were carefully dissected and removed. Specimen were fixed in 4% paraformaldehyde overnight, decalcified in 20% ethylenediamine tetraacetic acid for 3 weeks, and embedded in paraffin. After deparaffinization and hydration, endogenous peroxidase was blocked with 1% hydrogen peroxide.
for 10 min. Nonspecific binding was blocked by incubation in phosphate-buffered saline containing 10% normal serum (same species as secondary antibody) for 10 min. For immunostaining, commercially available polyclonal goat anti-BMPR IA, IB, and II antibodies were used (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Sections were incubated with these primary antibodies (dilution 1:100 in phosphate-buffered saline with 1% of normal serum) for 1 h in a humidified chamber. For negative controls, the primary antibody was omitted. A biotinylated anti-goat antibody was used as secondary antibody (Vector Labs, Burlingame, CA, USA) for 1 h. Sections were stained using the avidin-biotin complex method for 30 min, followed by DAB-peroxidase revelation. Finally, sections were counterstained with hematoxylin and mounted.

According to data provided by the manufacturer, the primary antibodies used in the present study recognize mouse, rat, and human BMPR. Therefore, we tested whether these antibodies also recognize rabbit BMPR to verify whether the observed staining pattern represented BMPR specific signal. According to the manufacturer’s instructions, 100 µL of goat BMPR-blocking peptide at a concentration of 200 µg/mL was inserted in a speedvac (Savant Inc., Farmingdale, NY, USA) to obtain the blocking peptide in a powder form. This was mixed with 20 µL of primary antibodies (concentration of 200 µg/mL) and pre-incubated overnight at 4 °C. Then the same protocol as for the sections without the blocking peptides was used. When sections were treated as described above, no staining was evident. Thus, the antibodies used in the present study recognized rabbit BMP receptors.

The number of cells expressing BMP receptors protein was assessed by cell counting. Chondrocytes, and osteoblastic and fibroblastic cells were identified morphologically. These analyses were performed separately for the callus region and the central region containing the fibrous interzone.

### Table 3
Densitometry and histomorphometry results

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Sham</th>
<th>n</th>
<th>Carrier</th>
<th>n</th>
<th>80 µg OP-1</th>
<th>n</th>
<th>800 µg OP-1</th>
<th>n</th>
<th>2000 µg OP-1</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Densitometry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone mineral content (g)</td>
<td>10</td>
<td>0.67 ± 0.04</td>
<td>6</td>
<td>0.67 ± 0.03</td>
<td>8</td>
<td>0.73 ± 0.06</td>
<td>8</td>
<td>0.72 ± 0.07</td>
<td>8</td>
<td>0.78 ± 0.06</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>Areal bone mineral density (g/cm²)</td>
<td>10</td>
<td>0.49 ± 0.03</td>
<td>6</td>
<td>0.44 ± 0.03</td>
<td>8</td>
<td>0.50 ± 0.03</td>
<td>8</td>
<td>0.55 ± 0.03</td>
<td>8</td>
<td>0.54 ± 0.03</td>
<td>0.15</td>
<td></td>
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<tr>
<td><strong>Histomorphometry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone volume/tissue volume (%)</td>
<td>3</td>
<td>42 ± 4</td>
<td>3</td>
<td>37 ± 8</td>
<td>4</td>
<td>44 ± 5</td>
<td>3</td>
<td>45 ± 2</td>
<td>3</td>
<td>46 ± 6</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>Fibrous volume/tissue volume (%)</td>
<td>3</td>
<td>10 ± 5</td>
<td>3</td>
<td>3 ± 1</td>
<td>4</td>
<td>7 ± 2</td>
<td>3</td>
<td>7 ± 1</td>
<td>3</td>
<td>4 ± 1</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Cartilage volume/tissue volume (%)</td>
<td>3</td>
<td>2.5 ± 1.6</td>
<td>3</td>
<td>1.0 ± 1.0</td>
<td>4</td>
<td>1.0 ± 0.9</td>
<td>3</td>
<td>0.2 ± 0.1</td>
<td>3</td>
<td>1.1 ± 0.5</td>
<td>0.59</td>
<td></td>
</tr>
</tbody>
</table>

*Note: Values are mean ± SE.*
Statistical analysis

Differences between treatment groups were tested for significance using analysis of variance (ANOVA). Throughout, a $P$ value of less than 0.05 was considered significant.

Results

All animals tolerated the surgical procedure well. However, two rabbits subsequently developed complications (displacement of the fixator secondary to broken pins and fracture at the proximal pin site in one case each) and had to be sacrificed before the end of the experiments. Both were excluded from the analysis. Table 1 gives the number of rabbits in each group and the amount of analyses that were performed.

Radiological examination at the end of the study period showed that complete bridging of the distracted zone with bone occurred only in the group that received 2000 µg of OP-1 (Fig. 3). In all the other groups, there was a narrow radiolucent line at the middle of the distracted zone. In none of the specimen could cortices be identified in the distracted zone.

Caliper measurements of the callus showed no significant differences between the five groups, as shown in Table 2. BMC and areal BMD of the distracted zones showed a dose dependent trend to increase (Table 3), which did not reach statistical significance. Histomorphometric analysis also did not yield significant differences between treatment groups (Table 3).

Results of mechanical testing are shown in Table 4. Two specimen—one from Group I and another from Group II—already fractured during dissection despite careful handling, suggesting that they had not consolidated. One specimen from Group V was angulated and was also excluded from

### Table 4

Results of biomechanical testing

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Ultimate load-to-failure (Newtons)</th>
<th>Energy-to-failure (N-mm)</th>
<th>Stiffness (N/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Std. deviation</td>
<td>Mean</td>
</tr>
<tr>
<td>I. Sham</td>
<td>9</td>
<td>801</td>
<td>183</td>
<td>533</td>
</tr>
<tr>
<td>II. Carrier</td>
<td>5</td>
<td>939</td>
<td>314</td>
<td>723</td>
</tr>
<tr>
<td>III. 80 µg OP-1</td>
<td>6</td>
<td>925</td>
<td>327</td>
<td>543</td>
</tr>
<tr>
<td>IV. 800 µg OP-1</td>
<td>6</td>
<td>920</td>
<td>324</td>
<td>615</td>
</tr>
<tr>
<td>V. 2000 µg OP-1</td>
<td>6</td>
<td>1114</td>
<td>257</td>
<td>887</td>
</tr>
<tr>
<td>Contralateral nonoperated tibiae</td>
<td>32</td>
<td>1749.3</td>
<td>164</td>
<td>1938.81</td>
</tr>
</tbody>
</table>

### Table 5

Results of BMP receptors quantification at weekly intervals

<table>
<thead>
<tr>
<th>Protein</th>
<th>Week</th>
<th>Center</th>
<th>Callus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Osteoblastic cells (preosteoblasts)</td>
<td>Chondrocytes</td>
</tr>
<tr>
<td>BMPR IA</td>
<td>1</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>++</td>
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<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+/-</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BMPR IB</td>
<td>1</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>+++</td>
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<td>++</td>
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<tr>
<td></td>
<td>4</td>
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<td>++</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+/-</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BMPR II</td>
<td>1</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>+++</td>
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<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Note: Weeks 1 to 3: distraction phase. Weeks 4 to 6: consolidation phase. –, No positive staining; +/-, presence of; +, less than 1/3 of cells positive; ++, 1/3 to 2/3 of cells positive; ++++, more than 2/3 of cells positive.
the results. Most specimens (65% or 20/31) fractured through the distracted zone. It is interesting to note that two specimens in the 2000 mg OP-1 group fractured outside the distracted zone, suggesting that their tensile strength was greater than the values obtained.

The load displacement curve patterns were very uniform, with a small toe region at first then followed by a long straight rise exhibiting a purely elastic behavior. The intact nonoperated samples achieved significantly higher load-to-failure as shown in Table 4.

There was a trend in the groups receiving increasing doses of OP-1 to have higher values for ultimate load-to-failure and energy-to-failure than the sham group. In the group receiving 2000 μg OP-1, ultimate load-to-failure was 40% higher than in the sham group. However, group differences did not achieve statistical significance by ANOVA. It is interesting to note that the carrier group had higher values than both groups with lower doses of OP-1 (80 and 800 μg groups).

The expression of BMPR IA, IB, and type II was studied at weekly intervals (Table 5). At the start of distraction, only chondrocytes showed weak staining for all three receptors. The most intense staining was evident during the second week of distraction (Fig. 4), mostly in chondrocytes and fibroblasts. Toward the end of distraction and during the consolidation phases, staining became gradually less intense. As a whole, staining was more intense in cells with a chondrocytic and fibroblastic appearance.

**Discussion**

In this study we examined whether a single local injection of OP-1 given at the end of the distraction period could speed up bone consolidation in DO. Indeed, rabbits receiving 2000 μg of OP-1 had the highest values in densitometric and histomorphometric parameters of bone mass as well as in biomechanical testing. Ultimate load-to-failure was 40% higher in these animals than in the sham group. It is thus possible that OP-1 at a high dose has a beneficial effect in
DO. Nevertheless, this putative beneficial effect was not large enough to clearly override the biological variability inherent in such experiments. Consequently, the differences between groups did not reach statistical significance with the number of samples used in this study. In the only other report on the use of BMPs in DO that we were able to find [41], positive results were obtained with locally applied 75 μg recombinant BMP-2 (by injection or absorbable collagen sponge) in a rabbit model. In that study, however, the authors intentionally increased the distraction rate to 2.0 mm a day (instead of the standard 1.0 mm that we used in our study) in order to create a poor ossification model, thus rendering both studies incomparable.

Local application of osteoinductive agents such as OP-1 by means of an injection is an appealing miniinvasive technique to accelerate new bone formation in DO. As shown in Table 4, however, the carrier group had higher biomechanical values than both the 80 and 800 μg groups, suggesting that the mechanical trauma induced by the needle used for the injection had some effect on stimulation of new bone in the distracted zone. This may have caused an inflammatory response leading to release of certain osteogenic factors. This mechanical effect of the needle was also reported by Li et al. [41].

In earlier studies, we had observed high levels of expression of BMP-2, 4, and OP-1 during the distraction phase, with a rapid decline thereafter [35]. Therefore, we had hypothesized that the best time for exogenous injection of BMPs would be at the end of the distraction phase, when the endogenous expression of BMPs was decreasing. However, the present results showed that this approach required very high doses of OP-1, and even then there was no unequivocally positive effect on bone consolidation. This suggests either that OP-1 is not a good agent in the context of DO, or that the timing or mode of its application was not ideal.

Immunohistochemical analyses of BMPR expression were performed in order to explain the modest success of OP-1 injections at the end of the distraction period. Strong expression of BMPR IA, IB, and II was found during the early distraction phase which then gradually decreased. This pattern mimics the expression profile of OP-1 during DO [35]. It appears that the expression of both OP-1 and its receptors is related to the mechanical forces of distraction in DO—strong expression as long as the distraction is maintained and quick downregulation as soon as the distraction stops. Thus, OP-1 given at the end of the distraction period cannot have a marked effect, because only a small amount of receptor protein is present in the target tissue.

These receptor expression data suggest that OP-1 may be more effective when given at the start of distraction. Indeed, there is preliminary evidence from a rat model that local injection of OP-1 at the time of the osteotomy significantly enhances bone formation in the distracted zone, even when given at a dose that is two orders of magnitude lower than the maximal dosage used in the present study [42].

One limitation of this study is that the distribution of the injected material into the tissue was not evaluated. The amount of injected material (0.2 ml) represents about 1/15 of the total volume of the regenerate bone (about 3.0 ml, as shown in Table 2). This raises the question: what was the distribution of the injected material into the tissue? This is an important issue to consider as distribution may be a limiting factor such that, even if a small number of receptors are present, if all those receptors were bound by the ligand, perhaps an effect could be demonstrated.

In conclusion, OP-1 given at the end of the distraction period, as a single bolus, did not have a marked effect on bone consolidation, even when given at very high doses. Possibly, OP-1 could be more useful when applied early in the distraction phase.

Acknowledgments

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