Effects of bone morphogenetic protein 2 gene therapy on new bone formation during mandibular distraction osteogenesis at rapid rate in rabbits

Jie Long, MD, PhD, Peng Li, MD, PhD, Hong-ming Du, MD, PhD, Lei Liu, MD, PhD, Xiao-hui Zheng, MD, PhD, Yun-feng Lin, MD, PhD, Hang Wang, MD, PhD, Wei Jing, MD, PhD, Wei Tang, MD, PhD, Wei-hui Chen, MD, PhD, and Wei-dong Tian, MD, PhD, Chengdu and Fuzhou, China

SICHUAN UNIVERSITY, CHENGDU CHILDREN’S SPECIAL HOSPITAL, AND FUJIAN MEDICAL UNIVERSITY

Objective. We investigated the effect of recombinant human bone morphogenetic protein 2 (rhBMP-2) on new bone formation during rapid-rate mandibular distraction osteogenesis. We also explored the feasibility of using local BMP-2 gene therapy to compensate for bad callus formation caused by a rapid distraction rate.

Study design. Bone marrow mesenchymal stem cells (MSCs) from Japanese rabbits were transfected with adenovirus (adv)–BMP-2. The right mandibles of the rabbits were distracted after corticotomy. The distraction rate in group A was 0.8 mm/d. The distraction rate in group B was 2.4 mm/d, and the distraction gap was injected with adv-lacZ-transfected bone marrow MSCs. The distraction rate in group C was 2.4 mm/d, and the distraction gap was injected with adv–BMP-2–transfected bone marrow MSCs. New generation bone tissue in the distraction gap was analyzed by plain radiograph examinations, microfocus computerized tomography (micro-CT) examinations, and biomechanical tests at weeks 2, 4, and 8 of the consolidation period.

Results. Radiographic and micro-CT examinations showed a better bone quality in group C compared with group A at weeks 2 and 4 of the consolidation period. There was no obvious new bone formation in group B. The trabecular parameters (trabecular thickness, trabecular number, volumetric bone mineral density at tissue, and bone volume fraction) were significantly higher in group C than in group A at weeks 2 and 4. At week 8, no significant difference were detected for all parameters except trabecular number between groups A and C. All biomechanical stress parameters were significantly higher in group C than in group A at week 4, and only peak stress was significantly different at week 8.


Distraction osteogenesis (DO) is a surgical technique used to treat skeletal deformities or defects by lengthing or expanding the skeleton. This is achieved by exerting a specified distraction force on bone segments where the periosteum and soft-tissue attachment are still preserved. DO has become an effective tool in the treatment of different kinds of craniofacial deformities and bone defects, such as craniofacial congenital malformation, ankylosis of TMJ, and bone defects after tumor resection. However, the major disadvantage of this method is the long distraction and consolidation period, which could result in severe complications during the distraction process, such as local infections. This has limited the effective clinical application of DO. The major objectives in current DO research focus on acceleration of new bone formation and shortening the treatment period.

The ideal distraction rate in DO to generate good bone formation has been found to be 1 mm/d; however, this relatively slow distraction rate has been reported to cause pain and an increase in the frequency of local infections during the long distraction period. The application of a rapid distraction rate may be an effective method to shorten the distraction period, but rapid distraction rates are thought to lead to defects in new
bone formation in the distraction gap.\textsuperscript{5-8} Compensating for the detrimental effects of a rapid distraction rate is therefore a key challenge.

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor \(\beta\) superfamily. BMP-2, an important member of this family, has been demonstrated to have highly effective osteogenic activity. However, due to the short-term bioactivity and rapid diffusion in vivo of direct recombinant protein delivery, the osteoinductive effects of BMP-2 may not be maximized in vivo. To this end, some recent studies have demonstrated that the use of recombinant human BMP-2 (rhBMP-2) local gene therapy can promote the quality and quantity of new bone formation in vivo by maintaining high expression of BMP-2 in vivo.\textsuperscript{9-13}

In the present study, we used an ex vivo gene therapy strategy where rhBMP-2 gene–modified bone marrow mesenchymal stem cells (MSCs) were injected into the mandibular distraction gap in a rabbit model with a rapid distraction rate. We analyzed the effects of BMP-2 gene therapy on new bone formation during mandibular distraction osteogenesis with different distraction rates. We determined the feasibility of using local rhBMP-2 gene therapy to compensate for bad callus formation caused by rapid distraction rate. An additional aim of this study was to shorten the distraction and consolidation period in mandibular distraction osteogenesis by using gene therapy.

MATERIAL AND METHODS

Animals

Thirty-six male Japanese rabbits, each weighing \(\sim 2,000-2,500\) g, were used in this study. All of the animals were kept in a standard animal holding facility under veterinary supervision at the Laboratory Animal Center of the West China Hospital, Sichuan University. All of the animal protocols were approved by the Chinese Academy of Agricultural Science.

MSC isolation and culture

Rabbit bone marrow stromal cells were isolated from the right femur using density-gradient centrifugation. Femur bone marrow (\(\sim 2\) mL) was cultured in alpha minimum essential medium supplemented with 15% fetal bovine serum (Gibco BRL, USA) and antibiotics (penicillin 80 U/mL, streptomycin 0.1 mg/mL). The cell suspension was incubated at 37\(^\circ\)C with 5% humid carbon dioxide. After \(\sim 10\) days of primary culture, the cells were passaged. Passage 2 cells were used for gene transfections.

Transfection with adenovirus (adv)–hBMP-2 and adv-lacZ

Adenovirus–hBMP-2 and adv-lacZ were kind gifts from Dr. You-Chao Tang at the biomedical engineering laboratory of Sichuan University. Passage 2 bone marrow stromal cells were prepared as described above and cultured to 90% confluence. The cells were infected overnight with either adv–hBMP-2 or adv-lacZ lysate at 37\(^\circ\)C (100 multiplicity of infections [MOIs]) and cultured in alpha minimum essential medium.

Determination of BMP-2 in culture after transfection

Enzyme-linked immunosorbent assay (ELISA) was used to detect the secreted BMP-2 protein in media of adv-hBMP-2– and adv-lacZ–transfected MSC cultures after 2, 4, or 6 days of transfection. ELISA was performed using the Quantikine ELISA kit for BMP-2 (R&D, USA) according with the manufacturer’s recommendations.

Design of the distraction device

A customized unilateral distraction device was designed for this study. The distraction device was made of titanium alloys and consisted of a retention plate, an activation rod, and a guide rod. The activation rod was positioned extraorally and 1 clockwise full turn of the activation rod resulted in 0.4 mm separation between the 2 fixation arms. The maximum elongation capacity allowed by this distraction device was \(\sim 12\) mm.

Anesthesia, surgical procedure, postoperative care, and distraction

All animals underwent the same surgical procedure. Animals in a supine position were anesthetized with an intramuscular injection of xylazine (4 mg/kg), acepromazine (1.5 mg/kg), and ketamine (15 mg/kg). A submandibular skin incision was made longitudinally to expose the edentulous mandibular body between the anterior teeth and the first premolar. The corticotomy was performed immediately anterior to the first premolar with a pneumatic fissure-cutting bur. The distractor was placed along the plane perpendicular to the corticotomy cut fissure and fixed with 2-mm-diameter titanium screws; the wound was irrigated with saline solution. Finally, the periosteum, subcutaneous tissue, and skin were repositioned and closed in layers with 3-0 silk sutures, and the activation rod was placed submandibularly.

After the operation, the animals were intramuscularly administered with an antibiotic (5 mg cefoparazone sodium) once daily for a week. The skin wound was thoroughly cleaned with iodine tincture every day to prevent infection. After a 7-day latency period, the animals were distracted according to their groups (details in next section).
Subject groups and distraction procedure

The 36 rabbits in the study were randomly divided into 3 groups of 12 rabbits each. The normal distraction group (group A) was subjected to a distraction rate of 0.8 mm/d for 12 days. The rapid distraction group (group B) was subjected to a distraction rate of 2.4 mm/d for 4 days, and these animals were injected with bone marrow MSCs transfected with adv-lacZ marker gene. The rapid distraction and gene therapy group (group C) was subjected to a distraction rate of 2.4 mm/d for 4 days and these animals were injected with bone marrow MSCs transfected with adv–hBMP-2. Distraction was performed twice daily in all 3 groups after a 7-day latency period. The total elongation was ~10 mm in all animals.

Gene therapy

MSCs transfected with adv–hBMP-2 were harvested 2 days after transfection. They were washed with phosphate-buffered saline solution and diluted in physiologic saline to a concentration of $1 \times 10^7$ cells/mL. Rabbits in group C were injected with $1 \times 10^7$ of this cell suspension (1.0 mL) directly into the distraction gap on the last day of the distraction period. Similarly, rabbits in group B were injected with the same dose of MSCs transfected with adv-lacZ. The animals were held in rigid fixation until they were killed.

Assessment methods

Four rabbits in each group were killed at week 2, week 4, and week 8 of the consolidation period. Mandibular specimens including the new-generation bone were obtained and were assessed with plain radiographic examinations, microfocus computerized tomography (micro-CT) and biomechanical testing methods.

Plain radiographic examinations. Soft-tissue was excised from the dissected mandibles. Each mandible was placed on an occlusal film with the lingual side touching the film. Lateral plain radiography was performed by an x-ray unit (Gendex Den Somat, Milano, IL) under standard conditions of 70 kV and 8 mA.

Micro-CT. After plain radiographic examination, the distracted new bone tissues and a 3–6-mm section of the neighboring normal bone were subjected to morphologic and quantitative examination using a Micro-CT 80 scan machine (Scanco Medical, Bassersdorf, Switzerland). Each specimen was placed in a midsize sample holder with the mandible body plane vertical to the x-ray tube. Images were acquired with the x-ray tube operating at an energy level of 60 kV and 180 µA. The serial scanned images of each specimen were analyzed on the computer using the recommended software (Scanco Medical). The total area of the distraction gap was outlined on each scanning image as the region of interest. Trabecular parameters of interest were calculated using the Analyze visualization software (Scanco Medical). The final parameters obtained included trabecular thickness (Tb.Th), trabecular number (Tb.N), volumetric bone mineral density at tissue (tBMD), bone volume fraction (BVF), trabecular spacing (Tb.Sp), and structure model index (SMI).

Biomechanical testing (3-point bending test). Because new-generation bone at week 2 in groups A and C was not mature enough to bear the machining process, only week 4 and week 8 new-generation bone were subjected to biomechanical testing. Soft tissue was excised from the dissected mandibles, and bone tissue of the distraction gaps in group A and group C were machined into $15 \times 2 \times 1.5$ mm standard specimens. During the cutting process, the specimens were moistened continuously with 0.145 mol/L NaCl solution. The specimens were then stored frozen at $-20^\circ$C and were thawed to room temperature immediately before testing. All specimens were subjected to 3-point bending testing using a WD-10A electronic materials testing machine (Guangzhou Testing Instruments, Guangzhou City, China). The specimens were positioned and fixed on the base plate, and the 3-point bending load was applied at rate of 1 mm/min until the specimen ruptured. During the test, the displacement and load data of every specimen were recorded, and material mechanical testing parameters, including slope of the stress-strain curve, peak stress, bending modulus, and energy to failure were obtained and analyzed using a personal computer.

Statistical analysis

Groups were compared using the 1-way analysis of variance and Student-Newman-Keuls post hoc q test with the SPSS version 11.0 software (SPSS, Chicago, IL, USA). Statistical significance was defined as $P < .05$.

RESULTS

Expression of BMP-2 in the culture supernatant of MSCs

The concentration of BMP-2 in the culture supernatant increased gradually with the culture time. BMP-2 levels were significantly higher in culture supernatant of adv–hBMP-2–transfected MSCs compared with adv-lacZ–transfected MSCs ($P < .05$; Table I).

Clinical outcome

There was no obvious new bone formation in the distraction gaps of specimens in group B. Ideal new bone formation could be seen in both group A and group C. At the end time of the experiment, the distraction devices fixation were still rigid and well positioned in all of the rabbits except in group B. We thought the bad new bone formation could have led to the distraction
In group B, device fixation failure was observed. In group A and group C, obvious crossbite and malocclusion of the lower incisor were observed. The right mandibles of rabbits in groups A and C were lengthened to ~10 mm.

Plain radiographic examinations

The distraction gaps in group B rabbits did not show ideal new bone formation, except a little incomplete

Table 1. Measurement of BMP-2 in culture supernatants of transfected cells (pg/mL)

<table>
<thead>
<tr>
<th></th>
<th>Day 2 ± SD</th>
<th>Day 4 ± SD</th>
<th>Day 6 ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSCs transfected with adv–hBMP-2</td>
<td>852 ± 35*</td>
<td>2,820 ± 127*</td>
<td>4,120 ± 342*</td>
</tr>
<tr>
<td>MSCs transfected with adv-lacZ</td>
<td>82 ± 12</td>
<td>101 ± 14</td>
<td>145 ± 21</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD.

*Significant difference between the 2 groups (P < .05).

Fig. 1. Lateral radiographic view of rabbit hemimandibles in the consolidation period. A, group A at week 2; B, group C at week 2; C, group A at week 4; D, group C at week 4; E, group A at week 8 F, group C at week 8; G, group B at week 2.
union around the inferior mandibular border at week 2 (Fig. 1, G). Group A and group C animals showed partial union except for the central zone in the distraction gap, with more obvious radiopacity in group C animals at week 2 (Fig. 1, A and B). The distraction gap in group A and group C animals showed more mature new bone formation, higher radiodensity, and higher radiopacity, especially in the central zone of the distraction gap at week 4 compared with week 2 (Fig. 1, C and D). At week 8, the radiograph images of group A and group C specimens were almost identical to each other. The distraction gaps showed good mature forma-

Fig. 2. Micro-CT images of the middle section in the distraction gap at the transverse plane in the consolidation period. A, group A at week 2; B, group C at week 2; C, group A at week 4; D, group C at week 4; E, group A at week 8; F, group C at week 8.
Data are expressed as mean ± SD.

*Significant difference between group C and group A (P < 0.05).

Table II. Changes in trabecular parameters of groups A and C at week 2 of consolidation

<table>
<thead>
<tr>
<th>Group</th>
<th>Tb.Th (mm)</th>
<th>Tb.N (mm⁻¹)</th>
<th>tBMD (mg/mm²)</th>
<th>BVF (%)</th>
<th>Tb.Sp (mm)</th>
<th>SMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.063 ± 0.005</td>
<td>0.337 ± 0.017</td>
<td>137.5 ± 7.1</td>
<td>7.50 ± 0.54</td>
<td>0.815 ± 0.026</td>
<td>1.75 ± 0.08</td>
</tr>
<tr>
<td>C</td>
<td>0.076 ± 0.007*</td>
<td>0.417 ± 0.048*</td>
<td>185.1 ± 18.2*</td>
<td>8.86 ± 0.29*</td>
<td>0.655 ± 0.667*</td>
<td>1.68 ± 0.08</td>
</tr>
</tbody>
</table>

Table III. Changes in trabecular parameters of groups A and C at week 4 of consolidation

<table>
<thead>
<tr>
<th>Group</th>
<th>Tb.Th (mm)</th>
<th>Tb.N (mm⁻¹)</th>
<th>tBMD (mg/mm²)</th>
<th>BVF (%)</th>
<th>Tb.Sp (mm)</th>
<th>SMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.234 ± 0.006</td>
<td>0.791 ± 0.03</td>
<td>607.4 ± 21.6</td>
<td>47.28 ± 0.50</td>
<td>0.440 ± 0.032</td>
<td>0.59 ± 0.07</td>
</tr>
<tr>
<td>C</td>
<td>0.246 ± 0.007*</td>
<td>0.901 ± 0.04*</td>
<td>682.5 ± 41.4*</td>
<td>56.21 ± 2.83*</td>
<td>0.390 ± .017*</td>
<td>0.49 ± 0.05*</td>
</tr>
</tbody>
</table>

Table IV. Changes in trabecular parameters of groups A and C at week 8 of consolidation

<table>
<thead>
<tr>
<th>Group</th>
<th>Tb.Th (mm)</th>
<th>Tb.N (mm⁻¹)</th>
<th>tBMD (mg/mm²)</th>
<th>BVF (%)</th>
<th>Tb.Sp (mm)</th>
<th>SMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.283 ± 0.008</td>
<td>1.070 ± 0.111</td>
<td>827.7 ± 9.86</td>
<td>64.26 ± 0.488</td>
<td>0.281 ± 0.010</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>C</td>
<td>0.291 ± 0.006</td>
<td>1.126 ± 0.099*</td>
<td>856.9 ± 8.38</td>
<td>64.42 ± 1.688</td>
<td>0.297 ± 0.014</td>
<td>0.39 ± 0.03</td>
</tr>
</tbody>
</table>

Table V. Three-point bending test results of group A and C

<table>
<thead>
<tr>
<th>Group</th>
<th>Week 4</th>
<th>Week 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Curve slope (N/mm)</td>
<td>Peak stress (MPa)</td>
</tr>
<tr>
<td></td>
<td>280.00 ± 20.20</td>
<td>43.65 ± 5.30</td>
</tr>
<tr>
<td>A</td>
<td>513.31 ± 31.30</td>
<td>102.74 ± 17.45</td>
</tr>
<tr>
<td>C</td>
<td>520.50 ± 24.10</td>
<td>112.74 ± 21.01</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD.

*Significant difference between group C and group A (P < 0.05).

Groups A and C looked similar at week 8. Trabecular bone was more mature and was remodeling to lamellar. Cortical bone could be clearly seen. There was no difference between the 2 groups at the level of mineralization (Fig. 2, E and F). We used the Scanco software to calculate the relative ultrastructure parameters of new bone trabeculae in groups A and C at different times of the consolidation period. We demonstrated significantly higher levels of the 4 parameters Tb.Th, Tb.N, tBMD, and BVF in group C compared with group A at week 2 (P < .05). However, Tb.Sp was lower in group C compared with group A (P < .05). We found no significant difference in SMI between the 2 groups (P > .05) (Table II).

Groups A and C significantly differed in the 6 trabecular parameters at week 4 (P < .05). Tb.Th, Tb.N, tBMD, and BVF values were significantly higher, whereas Tb.Sp and SMI were significantly lower in group C compared with group A (P < .05; Table III).

Tb.N was significantly higher at week 8 in group C compared with group A (P < .05), and no significant differences were detected for other parameters between group A and group C (P > .05; Table IV).

Biomechanical testing (3-point bending test)

Group C had significantly higher curve slopes, peak stress, bending modulus, and energy to failure values at week 4 (P < .05) compared with group A. However,
DISCUSSION
BMPs play an important role in cellular proliferation and differentiation during embryonic development. They also can induce the generation of new bone and cartilage from undifferentiated mesenchymal cells. Earlier studies showed that BMP-2 is the most important protein in this family, with specific spatial and temporal expression during the process of DO and plays an important role in bone formation. However, BMP-2 protein has a short half-life and easily diffuses in vivo, which limits its osteoinductive effect during the process of bone formation in vivo. Direct application of BMP-2 protein into the local region is therefore controversial, because the osteoinductive results were not ideal. Local gene therapy using rhBMP-2 could result in a high level of BMP-2 protein which persists in vivo and accelerates the process of bone formation, and regional gene therapy using adenoviral-mediated direct gene transfer of BMP-2 was shown to promote new bone formation during distraction osteogenesis. In the present study, we demonstrated an osteoinductive effect in mandibular distraction osteogenesis using rhBMP-2 gene therapy. Using plain radiographic examinations, micro-CT, and biomechanical testing, we showed that treatment with adv–hBMP-2–transfected MSCs resulted in greater and faster new bone formation when compared with treatment using adv–lacZ–transfected MSCs. Our data suggested that BMP-2 ex vivo gene therapy could promote new bone formation in DO. Because efficient transfection of MSCs with BMP-2 is key to this process, we used ELISA to demonstrate high levels of BMP-2 protein in culture supernatants of MSCs transfected with adv–hBMP-2, which indicated efficient transfection. We also used immunohistochemistry and reverse-transcription polymerase chain reaction (PCR) to demonstrate successful transfection of MSCs in vitro (data not shown, reported in concurrent paper).

The ideal distraction rate of DO has been reported to be 1 mm/d and a higher distraction rate shown to lead to fibrous tissue formation and delay of osteogenesis. The present results showed that our group B animals, subjected to a distraction rate of 2.4 mm/d, exhibited poor bone formation and nonunion in the distraction gap during the consolidation period after injection with bone marrow MSCs transfected with adv–lacZ. Our data suggested that 2.4 mm/d was too fast for bone formation in mandible DO in our experimental model. The normal group (group A) and the group that received rhBMP-2 gene therapy with a rapid rate (group C) both exhibited good bone formation at each time point of the consolidation period. Radiograph examination showed a gradual increase in the radiopacity in the distraction gap. Micro-CT and biomechanical testing showed a higher quality of new bone in group C compared with group A at corresponding time points, and ideal new bone formation were observed at week 8 of the consolidation period in the 2 groups. These results suggested that regional gene therapy using rhBMP-2–transfected MSCs played a significant role in promoting new bone formation of DO and compensated for the bad callos formation caused by a rapid distraction rate.

Traditional bone histomorphometry is based on tissue sections, but the ultrastructure of trabeculae, especially the anisotropy and connectivity, cannot be determined by observation of bone tissue sections. In addition to biochemical indicators, the most important index of bone strength is the ultrastructure, including the number, thickness, interval spacing, interlinkage, and orientation of trabeculae. In recent years, micro-CT has become a convenient and precise method to study the ultrastructure of trabeculae. We used micro-CT to precisely calculate the ultrastructure parameters of new bone trabeculae, and these results agreed with our results from the radiograph and biomechanical examinations. These data all suggested that regional BMP-2 gene therapy can accelerate the process of bone formation and compensate for the bad osteogenesis caused by distraction osteogenesis at rapid distraction rates.

The process of bone formation in DO is closely related to the consolidation time, and the most active period of bone formation is the early stage of the consolidation period. The critical factor during the process of gene therapy for DO is the time point when the BMP-2 gene is delivered into the gap. Because large numbers of osteogenic cells and endogenous growth factors accumulate at the site of bone formation at the early stages of bone formation, delivery of exogenous BMP-2 gene at this site, which can interact with these osteogenic cell and endogenous growth factors, may effectively promote bone formation. We used gene therapy to deliver rhBMP-2 at the end of the distraction period in the present study because importing the BMP-2 protein at an early stage of consolidation period is thought to most effectively promote osteogenesis. Our results demonstrating optimal bone formation after gene therapy suggested that the time of gene delivery was correct.

Bone marrow MSCs are the precursors of osteoblasts, adipocytes, myoblasts, and tendon cells. Even though some studies have shown that the simple application of MSCs significantly promoted osteogenesis, our data demonstrated that group B animals (injected with MSCs transfected with a marker gene) exhibited poor osteogenesis. However, group C ani-
mals (injected with MSCs transfected by BMP-2 gene) exhibited good bone formation, suggesting that simple application of MSCs could not compensate for bad osteogenesis caused by rapid distraction rate; however, MSCs transfected with BMP-2 effectively compensated for bad osteogenesis caused by a rapid distraction rate.

In conclusion, we demonstrated that gene therapy using rhBMP-2–modified MSCs promoted new bone formation during mandibular distraction osteogenesis, and it effectively compensated for the detrimental effect on new bone formation caused by a rapid distraction rate. This approach could make it possible to reduce the treatment time of DO in future.

REFERENCES


Reprint requests:
Prof. Wei-dong Tian, MD
West China College of Stomatology
Sichuan University
14, Section 3, Renminnan Road, Chengdu
China
dr.surg.weidongtian@hotmail.com