Defect Repair in the Rat Mandible With Bone Morphogenic Proteins and Marrow Cells

Objective: To investigate the ability of a bone growth factor mixture and bone marrow cells to repair a critical size defect of the rat mandibular body.

Design: Prospective, randomized controlled trial.

Subjects: Thirty-seven male Fischer rats.

Interventions: Critical size defects 4 mm in diameter were created in the left mandibular bodies of the rats. The defects were filled with a bone marrow cell suspension (group 1), a synthetic bone matrix consisting of bovine collagen and calcium hydroxyapatite cement (group 2), the matrix and marrow cells (group 3), the matrix with 100 µg of bone growth factor mixture (group 4), or the matrix with bone growth factor mixture and marrow cells (group 5). Animals were killed after 8 weeks, and the nondecalcified specimens were processed histologically. Specimens from group 1 were not processed because there was no grossly appreciable bone regeneration. Stereologic techniques were used to determine and compare the volume fractions and volume estimates of mature bone, new bone, osteoid, marrow, remaining cement, and fibrous tissue in each defect.

Results: Volumes of mature bone, new bone, and remaining cement did not differ significantly among the groups ($P = .30$ for mature bone, $P = .17$ for new bone, and $P = .34$ for cement). However, group 4 and 5 specimens contained significantly more osteoid and larger marrow spaces than did the group 2 and 3 specimens ($P < .001$ for both). The specimens in groups 2 and 3 contained significantly more fibrous tissue ingrowth than did those in groups 4 and 5 ($P < .001$).

Conclusion: The synthetic bone substitute containing bone growth factor mixture was effective in stimulating new bone and osteoid development in the rat mandibular model.

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RECONSTRUCTION OF craniofacial bony defects after trauma and ablative oncologic procedures, or in the repair of congenital anomalies, is a frequent surgical challenge. Autogenous bone grafting for reconstruction of craniofacial defects is limited by resorption, potential donor site morbidity, and, at times, insufficient quantities of donor bone that matches the structural and functional qualities of the defect. Homologous and heterologous bone grafts carry the risks of disease transmission, variable resorption, and potential activation of the host immune system. Although vascularized bone grafts carry less risk of resorption than free bone grafts and enable the transfer of larger quantities of tissue, they require increased technical expertise and additional surgical time. Refinements in tissue engineering techniques during the past decade, and in particular the regeneration of skeletal tissues, have fostered promise in the eventual application of these technologies in the treatment of human disease.

Several studies have demonstrated the ability of bone morphogenic proteins to stimulate repair of critical size mandibular defects and effect alveolar augmentation in animal models. In this study, we investigated the ability of a bone growth factor mixture (GF$_{\text{inj}}$, Sulzer Biologics, Wheat Ridge, Colo) and bone marrow cells to repair a critical size defect in a rat mandibular body model. We hypothesized that the presence of bone marrow, which is a rich source of osteogenic precursor cells, would increase bone deposition.

METHODS

Institutional guidelines for the humane use of laboratory animals were followed, and the Committee for the Humane Use of Animals, State University of New York Upstate Medical University, Syracuse, approved the study.
SURGICAL PROCEDURES

Thirty-seven syngenic Fischer 344 retired male breeder rats weighing an average ± SD of 417.9 ± 30.4 g were housed in the Department of Laboratory Animal Resources (DLAR), State University of New York Upstate Medical University, Syracuse, at a constant temperature of 24.5°C for 1 week before surgery. The animals were fed commercial rat chow and had access to food and water ad libitum. The rats were divided into 5 experimental groups: (1) those whose mandibular defects were filled with marrow cells (n=9); (2) those whose mandibular defects were filled with a collagen (lyophilized bovine type I tendon collagen=hydroxyapatite cement (HAC) (Bone Source; Stryker Leibinger, Portage, Mich) matrix (n=5); (3) those whose defects were filled with the collagen-HAC matrix and bone marrow cells (n=11); (4) those whose defects were filled with a matrix consisting of collagen treated with a bovine bone growth factor mixture (GFm) and HAC (n=4); and (5) those whose defects were filled with the collagen-GFm-HAC matrix and marrow cells (n=8).

The growth factor mixture is an osteogenic noncollagenous protein extract of bovine femurs that has been used to effect spinal fusion in several animal models. Its 2 major components are bone morphogenic protein 3 and transforming growth factor β1. Bone morphogenic proteins 2 through 7, as well as transforming growth factors β1 and β2, and fibroblast growth factor 1 are also present in the composite in much smaller quantities by mass (<1%). Other proteins present in the extract that probably do not contribute to tissue growth include ribosomal proteins S20, L6, and L32, and a protein related to hydrogenase of bovine femurs that has been used to gain weight postoperatively, and this weight gain was significant (preoperative mean weight, 417.9 ± 30.4 g; postoperative mean weight, 437.0 ± 34.6 g; P<.001).

HISTOLOGIC EXAMINATION

The hemimandibles were fixed in 10% neutral buffered formalin (Sigma-Aldrich Corp, St Louis, Mo) for 3 months. The specimens from group 1 animals were excluded from histologic processing and data analysis because there was no grossly appreciable bone regeneration. The remaining nondemineralized hemimandibles were dehydrated in graded ethanols and acetone under continuous negative pressure. They were similarly infiltrated with and embedded in polymethylmethacrylate. Sectioning was performed with a rotating diamond wafering saw (Buehler Ltd, Lake Bluff, Ill). The saw excursion was 650 µm for each section, with approximately half of this thickness being absorbed by the blade width. The sections were then mounted on plastic slides (Wasatch Histo Consultants, Inc, Winemucca, Nev), ground to a thickness of 100 µm or less, and polished. The sections were stained with counterstain (Surgipath Medical Industries, Inc, Richmond, Ill) and Masson trichrome stain (Surgipath Medical Industries, Inc). The sections were then dehydrated, mounted on a slide, and cover slipped.

STEREOLOGIC ESTIMATES

The volume fractions and volumes of osteoid, remaining cement, mature bone, new bone, marrow, and fibrous tissue were determined for the entire defect by means of design-based stereologic techniques. These techniques provide a statistically unbiased, quantitative estimate of the 3-dimensional composition of the defect and do not depend on any assumption regarding the tissue geometry. At least 2 sections were analyzed for each specimen (mean ± SD, 4 ± 1.1), with the number of fields and field size varying with the size and orientation of the defect. The cut edges of the native mandible were used to define the defect.

Data collection was performed in a blinded fashion with a ×4 objective. A video camera (Optronics LX450A CCD; Optronics, Inc, Muskogee, Okla) interfaced to a workstation (Silicon Graphics, Inc, Mountain View, Calif) was used to project images onto a monitor (Silicon Graphics, Inc). The stere-
logic data were collected with custom software that implemented an automated uniform random sampling protocol using computer-interfaced x- and y-axis stepping motors. The uniform random sample fields were distributed in a regular lattice pattern that was randomly positioned with respect to the tissue and were defined by a graphic overlay that included a regular point-counting lattice consisting of 108 points, with a distance of 0.12 mm between points. The volume fraction estimates \( V_{Y, \text{ref}} \) were made by means of test-point counting:

\[
V_{Y, \text{ref}} = \frac{\sum_{i=1}^{n} P_i}{\sum_{i=1}^{n} P_{\text{ref}}}
\]

where \( P_i \) is the number of lattice points hitting phase \( Y \) and \( P_{\text{ref}} \) is the number of points hitting the reference space. The total volume of the defect (\( \tilde{V} \)) was determined by means of the Cavalieri technique:

\[
\tilde{V} = T \cdot \frac{a}{p} \sum_{i=1}^{n} P_i
\]

where \( T \) is the distance between parallel sample planes, \( a/p \) is the area associated with each point, and \( P_i \) is the number of test points intersecting the defect in the \( i \)th section. Calculated areas for missing sections were filled with values of the means of adjacent sections. Total volumes (cubic millimeters) were obtained by multiplying the volume fraction of the respective defect compartment by the total defect volume.

STATISTICS

Data analysis was performed with BMDP statistical software (BMDP Statistical Software, Inc, Los Angeles, Calif). A standard factorial analysis of variance was used to analyze mean values for the defect volumes, volume fractions, and volumes of osteoid, cement, fibrous tissue, new immature bone, and mature bone. The first independent categorical variable was “BMP” (bone morphogenic protein), indicating the presence of the growth factor mixture, and the second independent categorical variable was “marrow cells.” Levene test was used to test for the equality of group variability. For variables that did not satisfy the assumption of equal group variances, a Brown-Forsythe test was substituted for the standard 2-way analysis of variance. A Bonferroni post hoc multiple analysis test was performed with SYSTAT software (SYSTAT Software, Inc, Richmond, Calif) to determine which pairs differed significantly. \( P \leq .05 \) was considered significant.

RESULTS

Volume fractions for new bone, osteoid, marrow, cement, and fibrous tissue are given in Table 1. Table 2 lists total defect volume, as well as volume estimates of new bone, osteoid, marrow, cement, and fibrous tissue.

DEFECT VOLUMES

Defect volumes did not differ significantly between the experimental groups (\( P = .11 \)). Defect volume means and SDs for groups 2, 3, 4, and 5 were 52.47±30.37 mm\(^3\), 42.02±18.24 mm\(^3\), 67.03±21.85 mm\(^3\), and 63.73±12.84 mm\(^3\), respectively (Table 2).

MATURE BONE

Very little of the bone matrices was replaced by mature bone, and none of the specimens in group 2 contained mature bone. There was no significant difference between the groups (\( P = .30 \)) (Table 2).

NEW BONE GROWTH AND OSTEOID VOLUMES

The specimens containing the growth factor mixture were characterized by defect healing with immature bone around the circumference of the defect that was contiguous with the cut edges of the native mandibular bone.

Table 1. Group Mean ± SD Volume Fractions for New Mature Bone, New Immature Bone, New Osteoid, Marrow, Remaining Cement, and Fibrous Tissue

<table>
<thead>
<tr>
<th>Group</th>
<th>Mature Bone</th>
<th>Immature Bone</th>
<th>Osteoid</th>
<th>Marrow</th>
<th>Cement</th>
<th>Fibrous Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>None</td>
<td>0.017 ± 0.016</td>
<td>0.002 ± 0.001</td>
<td>0.006 ± 0.010</td>
<td>0.208 ± 0.033</td>
<td>0.460 ± 0.018</td>
</tr>
<tr>
<td>3</td>
<td>0.002 ± 0.004</td>
<td>0.046 ± 0.042</td>
<td>0.025 ± 0.025</td>
<td>0.002 ± 0.003</td>
<td>0.269 ± 0.062</td>
<td>0.352 ± 0.062</td>
</tr>
<tr>
<td>4</td>
<td>0.0002 ± 0.0003</td>
<td>0.068 ± 0.052</td>
<td>0.112 ± 0.052</td>
<td>0.109 ± 0.104</td>
<td>0.267 ± 0.103</td>
<td>0.174 ± 0.065</td>
</tr>
<tr>
<td>5</td>
<td>0.009 ± 0.015</td>
<td>0.063 ± 0.036</td>
<td>0.077 ± 0.039</td>
<td>0.223 ± 0.085</td>
<td>0.214 ± 0.093</td>
<td>0.081 ± 0.038</td>
</tr>
</tbody>
</table>

Table 2. Group Mean ± SD Absolute Volumes for New Mature Bone, New Immature Bone, New Osteoid, Marrow, Remaining Cement, and Fibrous Tissue*

<table>
<thead>
<tr>
<th>Group</th>
<th>Defect Volume</th>
<th>Mature Bone</th>
<th>Immature Bone</th>
<th>Osteoid</th>
<th>Marrow</th>
<th>Cement</th>
<th>Fibrous Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>52.474 ± 30.373</td>
<td>None</td>
<td>1.098 ± 1.500</td>
<td>0.076 ± 0.085</td>
<td>0.152 ± 0.263</td>
<td>11.485 ± 7.846</td>
<td>24.251 ± 14.606</td>
</tr>
<tr>
<td>3</td>
<td>42.022 ± 18.240</td>
<td>0.094 ± 0.264</td>
<td>2.202 ± 2.374</td>
<td>1.371 ± 1.513</td>
<td>0.093 ± 0.148</td>
<td>10.555 ± 4.070</td>
<td>14.641 ± 9.968</td>
</tr>
<tr>
<td>5</td>
<td>63.730 ± 12.844</td>
<td>0.479 ± 0.731</td>
<td>4.047 ± 2.268</td>
<td>4.904 ± 2.681</td>
<td>13.636 ± 3.975</td>
<td>14.226 ± 7.689</td>
<td>5.402 ± 3.536</td>
</tr>
</tbody>
</table>

*All values are in cubic millimeters.
This bony shell enclosed a cavity that was filled with varying amounts of fatty marrow and remaining implant (Figure 1). The specimens that did not contain the growth factor mixture formed less new bone that did not extend much beyond the native mandibular cut edges (Figure 2). Although the average new bone growth for the groups with the growth factor mixture was greater than that for those without the growth factor mixture, these groups did not differ significantly (P = .17). The estimated volumes of total new bone for group 2 was 1.10±1.5 mm³, that for group 3 was 2.20±2.37 mm³, that for group 4 was 4.62±3.55 mm³, and that for group 5 was 4.04±2.27 mm³.

Osteoid volumes, however, were significantly greater in the specimens containing GFm (P < .001). Osteoid volumes were 0.08±0.08 mm³ and 1.37±1.51 mm³ for groups 2 and 3, respectively. For groups 4 and 5, these volumes were 6.79±1.42 mm³ and 4.90±2.68 mm³, respectively. Post hoc multiple analysis testing showed that group 4 differed significantly from groups 2 and 3 (P < .001 for both) and group 5 also differed significantly from groups 2 and 3 (P = .01 for both).

Marrow spaces were marked in the groups containing the growth factor mixture, and this difference was significant (P < .001). This accounted for the increased graft size in these specimens, in that this new growth in most cases extended well beyond the lingual and buccal surfaces of the native mandible. The estimated volumes of marrow spaces for groups 4 and 5 were 6.05±3.89 mm³ and 13.64±3.98 mm³, respectively. The volumes for groups 2 and 3 were 0.15±0.26 mm³ and 0.09±0.15 mm³, respectively. Group 4 differed significantly from groups 3 and 5 (P = .03 and P < .001, respectively), and group 5 differed significantly from groups 2 and 3 (P < .001 for both).

Cement and fibrous tissue volumes

Although the cement volumes did not differ significantly between the groups (P = .34 for remaining cement), the specimens without the growth factor mixture demonstrated extensive fibrous tissue ingrowth, with a fibrous tissue capsule surrounding the implants (P < .001 for fibrous tissue) (Figure 2). Cement volumes were 11.48±7.85 mm³ for group 2, 10.55±4.07 mm³ for group 3, 19.35±12.28 mm³ for group 4, and 14.22±7.69 mm³ for group 5. Fibrous tissue volumes were 24.25±14.61 mm³, 14.64±5.97 mm³, 12.60±8.45 mm³, and 5.40±3.54 mm³ for groups 2, 3, 4, and 5, respectively. Group 2 differed significantly from group 5 (P < .001). Figure 3 graphically depicts the volumes of the defect, mature bone, new bone, osteoid, marrow, and fibrous tissue for each experimental group.

Comment

The use of synthetic bone substitutes for repair of skeletal defects dates back to the 19th century with the use of plaster of paris to fill tuberculous cavities in long bones. The biocompatibility, osteoconductive, and osseointegrative properties of calcium-based alloplasts have been recognized for many years. Calcium hydroxyapatite has been in use for many years as an implant for treatment of long-bone fractures and alveolar augmenta-
The bone morphogenic proteins (except for bone morphogenic protein 1) are members of the transforming growth factor β superfamily of polypeptide growth factors. Approximately 20 BMPs have been identified and cloned. Recombinant BMPs have been shown to induce new bone formation in critical size mandibular defects in canine alveolar bone and rat mandibular angle models. A number of carriers for the BMPs have been used in several studies, including bovine microfibrillar collagen, bovine collagen–ceramic calcium hydroxyapatite composite alloplasts, porous ceramic calcium hydroxyapatite disks, and synthetic bioabsorbable polymers. The use of an implant containing collagen, recombinant BMP-2, and ceramic calcium hydroxyapatite beads was believed to result in improved alveolar ridge augmentation as compared with a collagen carrier and BMP-2 without the ceramic hydroxyapatite beads, demonstrating the ability of the calcium phosphate–containing implant to maintain bone volume. Toriumi and Robertson demonstrated the osteoinductive ability of recombinant BMP-2 to repair a full-thickness critical size segmental mandibular body defect in the canine model. This is the first study, to our knowledge, that used calcium HAC and a bone growth factor mixture (GFm) in a critical size defect in a rat mandibular body model. This synthetic bone substitute has the advantage of being easily molded.

Most of the specimens in our experimental groups contained relatively large amounts of remaining implant (volume fractions were 20.76%±3.32%, 26.90%±6.17%, 26.72%±10.26%, and 21.38%±9.29% for groups 2, 3, 4, and 5, respectively; Table 1), and these volume fractions were relatively consistent between groups. It is unclear whether the large amount of remaining cement in the defects was due to insufficient healing time or to isolation of the growth factor from osteoprogenitor cells by the HAC. The addition of collagen to the cement matrix was designed to enhance cellular infiltration. Extension of the postoperative healing time may have allowed for more complete resorption of the cement, and this is consistent with the findings of Breitbart et al, who used osteogenin (decalcified bone matrix) and tricalcium phosphate to repair rabbit calvarial defects. At 1 month, these investigators found 0.21% bone ingrowth and 38.76% tricalcium phosphate; at 3 months, 8.85% bone ingrowth and 32.86% tricalcium phosphate; and at 6 months, 22.33% bone ingrowth and 27.25% tricalcium phosphate. Differences in bone ingrowth and remaining implant material in that study did not reach significance until 6 months.

Surprisingly, the presence of the growth factor mixture in the current study did not result in significantly greater new bone formation, as has been demonstrated with the use of BMP-2 and BMP-7 in mandibular defect repair. Although the group 4 and 5 specimens grossly demonstrated more new bone formation than the other 2 groups, and the average new bone volumes were greater in groups 4 and 5 (4.62±3.55 mm³ and 4.04±2.27 mm³ vs 1.10±1.5 mm³ and 2.20±2.37 mm³, respectively), the averages between the groups only approached statistical significance (P=.17). Again, extension of the postoperative healing time may have borne out significant differences in new bone formation because the groups containing the growth factor mixture had significantly more osteoid present (P<.001). However, Linde and Hedner demonstrated complete bony bridging of a similar defect in the rat mandible after 12 days; Yoshida et al and Zellin and Linde demonstrated increased bony ingrowth in comparison with control subjects after 21 and 24 days, respectively; and Higuchi et al demonstrated increased bony ingrowth after 4 weeks. All of these studies used BMP-2, which may explain differences in healing times. Similarly, King et al were able to demonstrate increased alveolar bone ingrowth with BMP-2 after 10 days in the rat periodontal defect model, while Wikesjö et al had similar results after 8 weeks in the canine alveolar bone model. Giannobile et al were able to effect improved alveolar bone healing with bone morphogenic protein 7 after 8 weeks in the canine alveolar bone model.

Inadequate healing time may also explain the relative scarcity of mature bone in the specimens in the current study. The cut edges of the native mandible were difficult to distinguish in some sections, so that areas of new mature bone may have been underestimated. Fluorochrome labeling would have helped to make this distinction.

The development of large marrow spaces in the specimens containing the growth factor mixture accounted for the increased graft size in these specimens, as this new growth in most cases extended well beyond the lingual and buccal surfaces of the native mandible. The development of large marrow spaces has not been reported in any of the previous studies that used bone morphogenic proteins for defect repair or alveolar augmentation in the mandible. However, the fibrous tissue infiltration and replacement of the implants that did not contain the bone growth factor has been reported with all of the carriers used in these studies.

The rationale behind seeding of the implants in groups 3 and 5 with bone marrow cells was to determine whether the rate of bone deposition and matrix resorption could be influenced by the addition to the microenvironment of osteogenic precursor cells that were responsive to bone growth factors. In the groups without the growth factor mixture, the presence of bone marrow cells did not affect any of the measured variables. In groups 4 and 5, the presence of bone marrow cells resulted in larger marrow spaces, but had no effect on the other variables.

**CONCLUSIONS**

Few studies have evaluated new bone growth in mandibular defects implanted with bone substitutes containing growth factors with statistically unbiased, 3-dimensional morphometric analyses, and no studies have evaluated the effectiveness of the use of bone marrow cells in the repair of mandibular defects. We found that the presence of the growth factor mixture resulted in the for-
mation of significantly larger volumes of osteoid in the specimens implanted with this growth factor mixture. The defects filled with the GFm-containing implants also demonstrated the development of large, mature marrow spaces, which has not been previously reported. The presence of bone marrow cells did not appear to affect the rate of new bone formation, nor that of implant resorption, although it did result in larger marrow spaces in the GFm-implanted specimens. These results indicate that a synthetic bone substitute containing calcium HAC and a bone growth factor mixture is effective in the repair of mandibular defects.

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REFERENCES