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Comparison of BMP-2 and -4 for rat mandibular bone regeneration at various doses[1]

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Structured Abstract

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Objective – To compare mandibular bone regeneration with bone morphogenetic proteins-2 and -4 (BMP-2 and -4) at varying doses.

Study Design – Defects were created in the left hemi-mandibles of 82 Sprague–Dawley rats. The defects were filled with a hyaluronic acid polymer loaded with 0.01, 0.1, 1, or 10 μg of BMP-2 or -4. Control groups consisted of animals with unfilled defects, or with defects filled with the hyaluronic acid sponges loaded with growth factor dilution buffer. Animals were killed after 8 weeks, and the hemi-mandibles were analyzed histologically using stereologic techniques.

Results – Mandibles implanted with carriers containing 10 μg of BMP-2 or -4 differed significantly from controls in terms of new bone area ($p = 0.01$ and $p = 0.0001$, respectively). Marrow space development occurred in a dose-dependent fashion ($p < 0.0001$ for both growth factors), and this effect was more pronounced for BMP-2 at larger doses ($p < 0.0001$ at 1 and 10 μg doses). New bone areas and volumes did not differ significantly between the growth factors. While defects implanted with BMP-4 tended to have thicker cortical bone and more trabecular bone, at least partial defect bridging was achieved in a greater number of defects implanted with BMP-2 (47%) than with BMP-4 (35%).

Conclusion – Although similar areas and volumes of new bone were induced with BMP-2 and -4, differences were noted in the quality of bone generated with each growth factor. The results indicate a threshold dose for acute administration between 1 and 10 μg BMP-2 for bony union in this model, and ≥10 μg for BMP-4.

Significance – These findings suggest that differences in bone growth factor osteogenic potential deserve further study and may have an impact on the translation of osteoinductive protein therapy into clinical practice.

Key words: bone morphogenetic protein; healing; mandible; osteogenesis; repair

Introduction

Advances in osseous tissue bioengineering over the past decade have enabled the *in vivo* regeneration of living bone in many animal models, as well as the limited application of these techniques in orthopedic surgery. The bone morphogenetic proteins (BMPs) are the most potent bone growth factors known, and have been studied extensively in human and animal models of bone regeneration in the craniofacial and axial skeleton. However, the quantities of BMPs that have been used in most *in vivo* experimental models and clinical applications far exceed physiologic concentrations of the growth factors. The use of large quantities of these costly growth factors deserves consideration in this era of skyrocketing health care costs, and the development of methods utilizing quantities that are more physiologic may enable wider clinical application.

The BMPs (except for BMP-1) are members of the transforming growth factor-β superfamily of polypeptide growth factors. Approximately 40 BMP isoforms have been identified, and the BMPs are highly conserved between species (1, 2). These isoforms differ in their osteogenic potential, and their effects may be mitogenic, chemotactic, morphogenic, or apoptotic depending on the cell type to which the ligand is exposed, and the growth factor concentration (2). BMP-2 and -4 are essential for osteoblast and osteoclast formation, and have been shown to be the most strongly expressed BMPs in rodent models of mandibular fracture healing, as well as in new bone formation during distraction osteogenesis of the mandible (1,3–6). Although recombinant human BMP-4 has been reported to have greater osteogenic potential than BMP-2 and -7, both BMP-2 and -7 are currently used clinically for lumbar fusion and fracture repair in the axial skeleton (7).

In order to develop efficient, cost-effective systems for bone regeneration, it is necessary to determine minimal effective dose ranges of administration for BMPs. A greater understanding of the interactions of these and other biomolecules involved in osseous tissue development is also essential. This study compared new bone formation in the rat mandibular body model utilizing acute administration of various doses of BMP-2 and -4 in order to confirm effective *in vivo* dose ranges for these growth factors. We also sought to compare the osteogenic potentials of BMP-2 and -4 in the rodent mandible. We hypothesized that new bone deposition would occur with both growth factors in a dose-dependent manner. We further hypothesized that there would be no significant differences in osteogenic potential between BMP-2 and -4 at equivalent doses.

Materials and methods

Institutional guidelines for the humane use of laboratory animals were followed, and the Institutional Animal Care and Use Committee of the University of Kentucky at Lexington, Kentucky approved the study. Eighty-two Sprague–Dawley retired male breeder rats weighing an average of 503.93 ± 46.78 (standard deviation) g were housed in the Department of Laboratory Animal Resources (DLAR), University of Kentucky Medical Center at a constant temperature of 24.5°C for 1 week prior to surgery. The animals were fed commercial rat chow and had access to food and water *ad libitum*. Each left hemi-mandible was assigned to one of eight experimental groups: 1) 0.01 μg per defect of recombinant human BMP-2 (rhBMP-2), 2) 0.1 μg of rhBMP-2 per defect, 3) 1 μg of rhBMP-2 per defect, 4) 10 μg of rhBMP-2 per defect, 5) 0.01 μg of rhBMP-4 per defect, 6) 0.1 μg of rhBMP-4 per defect, 7) 1 μg of rhBMP-4 per defect, and 8) 10 μg of rhBMP-4 per defect. For group 1, n = 12, and for all other experimental groups, n = 10. The contralateral (right) hemi-mandibles were used as controls in 20 of the animals. The first control group (n = 11) consisted of right hemi-mandibles with unfilled defects. The second control group consisted of right hemi-mandibles implanted with hyaluronic acid sponges (MeroGel®; Medtronic Xomed Surgical Products, Jacksonville, FL, USA) loaded with growth factor dilution buffer (Control, n = 11).

Preparation of implants

The volume needed to fill the critical size defect determined the amount of carrier used. The hyaluronic acid sponges were cut into 4 mm × 4 mm squares for implantation. Prior to implantation, the carrier was soak-loaded for 15 min with either 50 μl of BMP dilution buffer (Control 1), or 50 μl of rhBMP-2 or rhBMP-4 solution (groups 1–8). This volume of liquid was found to be appropriate for filling the carriers without excess.

rhBMP-2 and -4 (Research Diagnostics, Inc., Flanders, NJ, USA) were suspended in a buffer (5 mM sodium glutamate, 2.5% glycine, 0.5% sucrose, 0.01% Tween 80, pH 4.5) at a concentration of 0.1 mg/ml. The growth factor solution was then diluted with the same buffer to deliver the appropriate amount of growth factor in each implant (0.01, 0.1, 1, or 10 μg per implant).

Surgical procedures

The rats were anesthesized with ketamine hydrochloride (60 mg/kg) and xylazine (5 mg/kg), administered intraperitoneally. Surgery was performed using aseptic technique. A linear incision was made through the skin, subcutaneous tissues and masseter muscle, paralleling the inferior border of the mandible. The buccal and lingual surfaces of the mandible were exposed with an elevator, and a 5 mm $\times$ 5 mm full-thickness defect was created in the body of the mandible, posterior to the root of the incisor. This ostectomy was performed with a high-speed drill and chilled normal saline irrigation, and did not interrupt mandibular continuity at the alveolus. Previous studies utilizing this experimental model confirmed that this critical size defect would not heal spontaneously.

The resulting defects were filled as previously described. The surgical wounds were closed in two layers (periosteum/muscle layer and skin layer) with 4-0 polyglactin suture. The animals were allowed to recover from anesthesia, and then returned to the DLAR for postoperative care where veterinarians supervised them. Buprenorphine 0.1 mg/kg was administered subcutaneously twice a day for the first 3 days postoperatively, and the rats were maintained on a diet of ground rat chow and water, to which they had access *ad libitum*.

One rat died intra-operatively, and another died 2 weeks postoperatively of unknown causes. There were no other complications. The surviving animals were killed 8 weeks postoperatively by lethal injection of pentobarbital sodium 150 mg/kg intraperitoneally, and the hemi-mandibles were harvested.

Histology

The hemi-mandibles were fixed in 10% neutral buffered formalin (Sigma Chemical Company, St Louis, MO, USA) for 1 week. The non-demineralized hemi-mandibles were dehydrated in graded ethanols and acetone under continuous negative pressure. Fifteen of the hemi-mandibles (one in group 1, four in group 3, three in group 4, four in group 7, two in group 8, and one in the control group) had to be excluded from further analysis because of histological processing errors. Thus a total of 85 hemi-mandibles remained to be analyzed. The specimens were infiltrated with and embedded in polymethylmethacrylate. Sectioning was performed with a rotating diamond wafering saw (Buehler, Lake Bluff, IL, USA). The saw excursion was 800 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, Winnemucca, NV, USA), ground to a thickness of 100 μm or less, and polished. The sections were stained with Sanderson's rapid bone stain (Surgipath Medical Industries, Richmond, IL, USA), and counterstained with acid fuchsin. This staining combination afforded sufficient contrast to distinguish bone, which stained pink, from osteoid (deep blue), and fibrous tissue (light blue). The remaining polyhyaluronic acid implant stained a dark, gray color.

Stereological estimates

The area fractions and volume estimates of osteoid, remaining cement, new bone, marrow, and fibrous tissue were determined for the entire defect using design-based stereological techniques. These techniques provide a statistically unbiased, quantitative estimate of the three-dimensional composition of the defect and do not depend on any assumption regarding the tissue geometry. At least one section was analyzed for each specimen (mean 3.73 $\pm$ 1.55) 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 using a 2$\times$ objective. A Nikon Eclipse E600 fluorescent microscope with a Spot Insight color digital camera was interfaced to a Dell Dimension L866r computer and used to project images onto a 15-inch Dell monitor. The stereological data were collected with custom software utilizing a uniform random (UR) sampling protocol with a stage micrometer. The UR 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. The area fraction estimates were made using test point counting. The total volume of the defect was determined using the Cavalieri technique (8). Calculated areas for missing sections were filled with values of the means of adjacent sections. Total volumes (mm^3) were obtained by multiplying the area fraction of the respective defect compartment by the total defect volume.

Statistical analysis

Data analysis was performed with Statview statistical software (SAS Institute, Cary, NC, USA). One-way analyses of variance (ANOVA) were used to identify differences in mean values for the defect volumes and area fractions of osteoid, cement, fibrous tissue, and new bone between the different doses of each growth factor (0.01, 0.1, 1, and 10 μg). The one-way ANOVA was also used to identify differences in mean values of volumes and area fractions between each growth factor (rhBMP-2 and -4) at identical doses. The Games/Howell *post-hoc* test was used to identify significant pair-wise differences. The importance of significant differences (effect size) was estimated by a Hayes ω^2 statistic. The effect size indicates the percentage of the total variances that is explained by the independent variable. An $0.01 < \omega^2 < 0.05$ indicates a small effect, an $0.06 \leq \omega^2 \leq 0.13$ indicates a medium effect, and an $\omega^2 \geq 0.14$ indicates a large effect. A p-value ≤ 0.05 was considered significant for all comparisons.

Results

Most of the animals continued to gain weight postoperatively, and the postoperative weights differed significantly from the preoperative weights (503.93 ± 46.78 g, postoperative 534.65 ± 53.00 g, $p = 0.0001$). The animals with bilateral hemi-mandibular defects did not differ significantly from the other experimental animals in terms of preoperative (518.98 ± 35.63 g vs. 498.42 ± 49.36 g for animals with unilateral defects) or postoperative weight (502.52 ± 72.75 g vs. 545.37 ± 39.87 g for animals with unilateral defects, $p = 0.25$).

Defect healing occurred in the mandibles implanted with the growth factors with woven bony shells enclosing large marrow spaces. Defects implanted with rhBMP-2-containing carriers had little trabecular bone growth, while specimens implanted with rhBMP-4-containing carriers tended to have more trabecular bone growth and thicker cortical bone (Figs 1 and 2). Partial defect bridging with new bone that did not extend to the most inferior extent of the defect occurred in 14 specimens, including two in group 1 (22%), four in group 3 (67%), one in group 5 (10%), two in group 6 (20%), one in group 7 (17%), and four in the control group containing the hyaluronic acid carrier (44%). Complete defect healing occurred in 17 experimental hemi-mandibles, and in none of the control

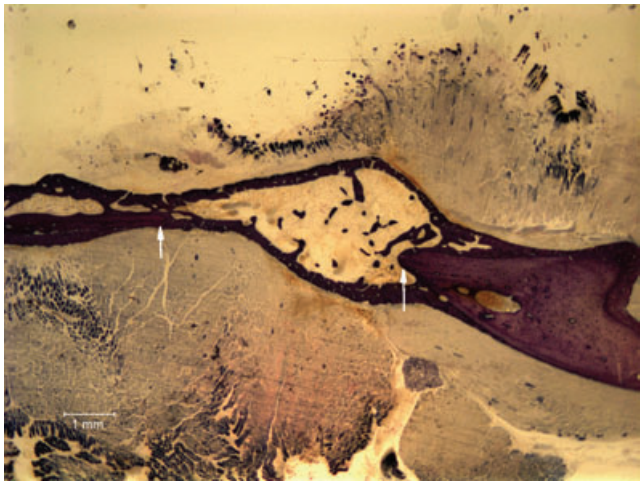

Fig. 1. Defect implanted with 1 μg rhBMP-2. Note new, immature, woven bone enclosing large marrow space. Arrows indicate defect edges (1× magnification, acid fuschin and Sanderson's rapid bone stain).

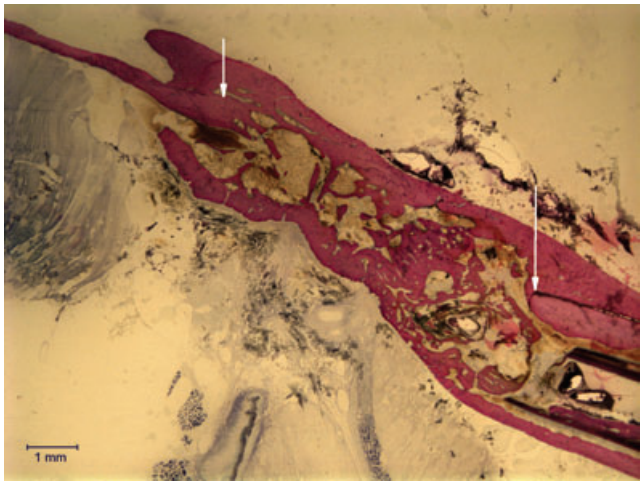

Fig. 2. Defect implanted with 0.1 μg rhBMP-4. Note smaller marrow space, thicker cortical bone, and increased area of trabecular bone in comparison with Fig. 1 (1× magnification, acid fuschin and Sanderson's rapid bone stain).

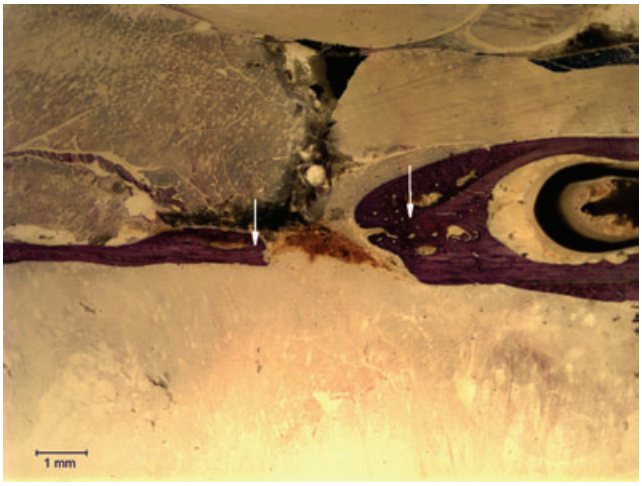

Fig. 3. Control mandible demonstrating limited new bone growth (1× magnification, acid fuschin and Sanderson's rapid bone stain).

specimens. Complete union occurred in one specimen in group 2 (10%), one in group 3 (17%), seven in group 4 (100%), one in group 7 (17%), and seven in group 8 (88%). Thus, at least 41% of the experimental defects had at least partial defect bridging with new bone. Most control defects demonstrated limited new bone growth at the defect edges, with collapse of adjacent musculature into the defect (Fig. 3).

Effects of growth factor dose

Tables 1 and 2 list average tissue volume estimates for defects implanted with rhBMP-2 and -4, respectively, with their standard deviations. Tables 3 and 4 list average area fractions for defects implanted with rhBMP-2 and -4, respectively. Dose increase did not have a statistically significant effect on new bone volume estimates in the experimental groups, although new bone volume tended to increase with increasing growth factor dose. The more sensitive area fraction analysis demonstrated a significantly larger fraction of new bone in the group containing 10 μg of rhBMP-4 when compared with other experimental groups with rhBMP-4-containing implants, and controls ($\omega^2 = 0.10$). Similarly, specimens implanted with 10 μg rhBMP-2 had larger new bone fractions than those implanted with 0.01 μg rhBMP-2, and controls ($\omega^2 = 0.07$).

Dose escalation had similar effects on marrow volume for both growth factors, as defects implanted with 10 μg of either growth factor had larger marrow volumes than those implanted with 0.01 and 0.1 μg doses, and control groups, but did not differ significantly from defects with the 1 μg dose ($\omega^2 = 0.53$ for rhBMP-2, $\omega^2 = 0.50$ for rhBMP-4). The marrow area fraction analysis for defects implanted with rhBMP-2 revealed differences identical to the marrow volume estimate analysis for rhBMP-2 ($\omega^2 = 0.58$), but the area fraction analysis for rhBMP-4 indicated that specimens implanted with 10.00 μg rhBMP-4 had larger marrow area fractions than all other groups ($\omega^2 = 0.57$).

Fibrous tissue volumes were significantly less in defects with 10.00 μg rhBMP-4 when compared with those implanted with 0.01 and 0.1 μg rhBMP-4, and control groups ($\omega^2 = 0.17$). Although experimental groups implanted with larger doses of rhBMP-2 tended toward smaller fibrous tissue volumes, the differences only approached statistical significance. The effect of rhBMP-2 dose on fibrous tissue in-growth was more demonstrable in the area fraction analysis, in which groups with larger doses of rhBMP-2 (1 and 10 μg) differed significantly from groups with smaller doses and controls ($\omega^2 = 0.26$). The area fraction analysis for fibrous tissue in specimens implanted with rhBMP-4 revealed that the 10 μg group differed from all other groups and controls ($\omega^2 = 0.28$).

Table 1. **Tissue volume estimates (mm^3) for groups implanted with rhBMP-2**

Dose	New bone	Osteoid	Marrow	Fibrous tissue	Remaining implant	Total defect volume
0.01 μg	1.58 ± 1.64	0.22 ± 0.39	0.46 ± 0.61	7.99 ± 4.99	2.08 ± 1.79	12.61 ± 7.61
0.1 μg	1.44 ± 1.25	0.07 ± 0.11	0.51 ± 0.63	7.39 ± 4.21	0.78 ± 0.51	10.34 ± 3.49
1 μg	1.59 ± 1.62	0.07 ± 0.12	3.00 ± 4.00	2.10 ± 2.52	0.47 ± 0.47	7.62 ± 7.04
10 μg	2.78 ± 0.96	0.04 ± 0.05	4.89 ± 2.10	2.74 ± 2.35	0.36 ± 0.39	10.90 ± 4.39
Control	1.12 ± 1.28	0.05 ± 0.09	0.38 ± 0.54	5.27 ± 3.74	3.37 ± 3.43	10.27 ± 8.08
Unfilled defect	1.23 ± 2.13	0.03 ± 0.04	0.12 ± 0.16	9.55 ± 8.00	0	12.45 ± 10.48
p	0.26	0.32	<0.0001	0.06	0.05	0.76

Table 2. **Tissue volume estimates (mm^3) for groups implanted with rhBMP-4**

Dose	New bone	Osteoid	Marrow	Fibrous tissue	Remaining implant	Total defect volume
0.01 μg	1.55 ± 1.23	0.08 ± 0.15	0.25 ± 0.19	8.20 ± 4.98	0.98 ± 0.89	10.43 ± 5.94
0.1 μg	1.91 ± 1.50	0.12 ± 0.19	0.61 ± 0.60	8.75 ± 6.99	0.97 ± 0.85	12.43 ± 8.92
1 μg	1.94 ± 1.96	0.17 ± 0.28	2.14 ± 2.94	7.62 ± 5.31	0.96 ± 1.03	12.88 ± 10.01
10 μg	2.38 ± 0.93	0	3.44 ± 1.60	0.66 ± 0.82	0.17 ± 0.24	6.68 ± 2.24
Control	1.12 ± 1.28	0.05 ± 0.09	0.38 ± 0.54	5.27 ± 3.74	3.37 ± 3.43	10.27 ± 8.08
Unfilled defect	1.23 ± 2.13	0.03 ± 0.04	0.12 ± 0.16	9.55 ± 8.00	0	12.45 ± 10.48
p	0.42	0.18	<0.0001	0.04	0.04	0.55

Table 3. **Tissue area fractions for groups implanted with rhBMP-2**

Dose	New bone	Osteoid	Marrow	Fibrous tissue	Remaining implant
0.01 μg	0.14 ± 0.18	0.02 ± 0.05	0.04 ± 0.06	0.65 ± 0.25	0.14 ± 0.14
0.1 μg	0.20 ± 0.24	0.01 ± 0.02	0.07 ± 0.10	0.63 ± 0.32	0.09 ± 0.13
1 μg	0.22 ± 0.14	0	0.36 ± 0.31	0.35 ± 0.32	0.06 ± 0.12
10 μg	0.30 ± 0.14	0	0.48 ± 0.22	0.18 ± 0.21	0.03 ± 0.05
Control	0.21 ± 0.28	0.02 ± 0.09	0.05 ± 0.08	0.43 ± 0.31	0.27 ± 0.27
Unfilled defect	0.09 ± 0.13	0	0.01 ± 0.03	0.75 ± 0.24	0
p	0.01	0.56	<0.0001	<0.0001	<0.0001

Table 4. **Tissue area fractions for groups implanted with rhBMP-4**

Dose	New bone	Osteoid	Marrow	Fibrous tissue	Remaining implant
0.01 μg	0.19 ± 0.20	0	0.03 ± 0.05	0.66 ± 0.21	0.12 ± 0.15
0.1 μg	0.20 ± 0.25	0.01 ± 0.02	0.06 ± 0.11	0.65 ± 0.31	0.08 ± 0.11
1 μg	0.15 ± 0.16	0.01 ± 0.03	0.15 ± 0.23	0.59 ± 0.33	0.10 ± 0.14
10 μg	0.36 ± 0.17	0	0.48 ± 0.23	0.14 ± 0.21	0.03 ± 0.06
Control	0.21 ± 0.28	0.02 ± 0.09	0.05 ± 0.08	0.43 ± 0.31	0.27 ± 0.27
Unfilled defect	0.09 ± 0.13	0	0.01 ± 0.03	0.75 ± 0.24	0
p	0.0001	0.50	<0.0001	<0.0001	<0.0001

Dose increase did not have an effect on remaining implant volume for defects implanted with a rhBMP-2-containing carrier, but there was a trend toward smaller implant volumes with increasing growth factor dose. Implant volumes were significantly less in the specimens containing 10 μg rhBMP-4 when compared with controls ($\omega^2 = 0.17$). Specimens implanted with 10 μg of either growth factor had smaller implant area fractions than those in the 0.01 μg dose group, and all experimental groups except the 0.01 μg dose group had smaller implant area fractions than the control ($\omega^2 = 0.15$ for rhBMP-2, $\omega^2 = 0.13$ for rhBMP-4). Dose increase did not have a significant effect on osteoid volume estimates or area fractions, or on total defect volumes.

Comparison of rhBMP-2 and -4

Both rhBMP-2 and -4 induced comparable volume estimates of new bone growth at all doses tested (Fig. 4). At the 10 μg dose, rhBMP-2 differed significantly from controls in terms of new bone volume ($\omega^2 = 0.17$), and both growth factors differed from controls in terms of new bone area fraction at this dosage ($\omega^2 = 0.18$). However, differences in marrow volume between the growth factors were apparent at

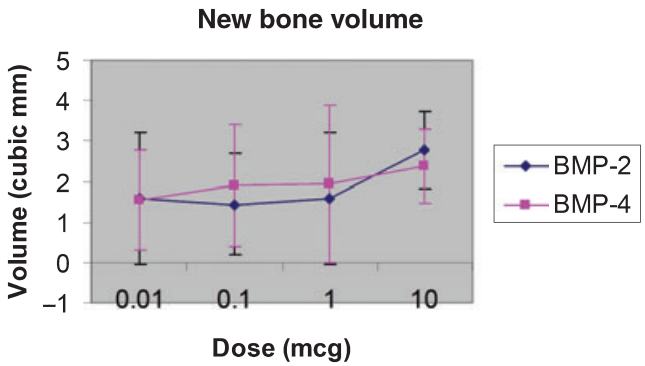

Fig. 4. Comparison of effects of dose escalation of rhBMP-2 and -4 on new bone growth.

the 1 and 10 μg doses, where implantation with rhBMP-2 consistently resulted in larger marrow spaces than implantation with rhBMP-4 ($p = 0.02$, $\omega^2 = 0.21$ and $p < 0.0001$, $\omega^2 = 0.72$, respectively, Fig. 5). Area fraction analysis demonstrated a similar difference in marrow space development between the two growth factors at the 1 μg dose ($p < 0.0001$, $\omega^2 = 0.34$), but at the 10 μg dose, both growth factors differed only from controls ($p < 0.0001$, $\omega^2 = 0.33$). Thus rhBMP-2 dose increase had a greater impact on marrow space development than did rhBMP-4 dose escalation. The relatively larger proportion of new bone in comparison with marrow in defects implanted with rhBMP-4 manifested as thicker cortical bone and larger volumes of trabecular bone than defects implanted with rhBMP-2.

Differences in fibrous tissue volume estimates between the growth factors were apparent only at the 10 μg dose, at which rhBMP-4 seemed to be more effective at limiting fibrous tissue in-growth than rhBMP-2 ($p = 0.01$, $\omega^2 = 0.25$). This finding was interesting, as at least partial defect bridging was achieved in a greater number of defects implanted with BMP-2 (47%) than with BMP-4 (35%). However, fibrous tissue area fractions were similar between the growth factors at the 10 μg dose, but did differ significantly from controls ($p < 0.0001$, $\omega^2 = 0.39$). At the 1 μg dose, the fibrous tissue area fraction was larger in rhBMP-4-containing defects than in those containing rhBMP-2 and controls ($p = 0.01$, $\omega^2 = 0.08$).

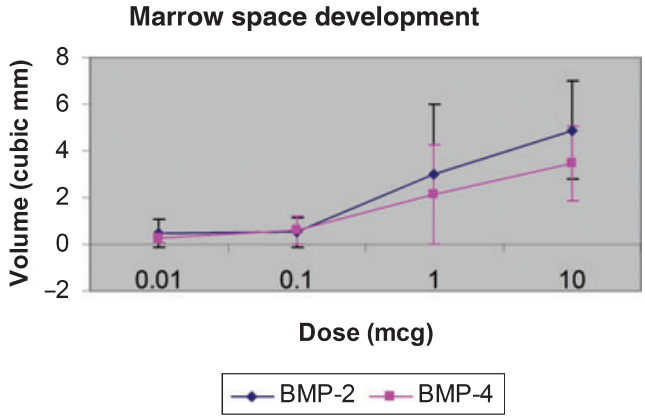

Fig. 5. Comparison of effects of dose escalation of rhBMP-2 and -4 on narrow space development.

Discussion

The translation of osteoinductive protein therapy into clinical practice is still in its early stages, and much needs to be elucidated about the interactions of biomolecules involved in bone healing and development. The use of osteoinductive proteins for the repair of head and neck defects is still in its experimental stages because characteristics typical of head and neck defects result in a suboptimal local environment for supporting tissue regeneration (i.e. vascular compromise because of radiation and tissue fibrosis, and potential oral cavity contamination). In addition, patients with oral cavity malignancies frequently have co-morbid conditions (malnutrition, alcohol- and tobacco-dependence, etc.) that negatively affect healing, and these patients are best served by one-stage reconstruction of post-ablative defects. Current osteoinductive protein therapy utilizes application of a single growth factor at supraphysiologic doses, which does not adequately replicate the normal bone healing process.

Bone healing is thought to occur in at least two to three phases, including an inflammatory phase, a chondrogenic phase, and an osteogenic phase. The inflammatory phase is characterized by a peak in interleukins-1β and -6 concentrations, with recruitment of inflammatory cells. This inflammation is thought to cause increased demineralization at the fracture site with resultant exposure of osteoinductive proteins in the organic bone matrix, including BMPs (9). Naturally occurring BMPs comprise 0.1% of total bone weight, indicating that they exist in concentrations on the order of tens of micrograms per milligram of bone (10). We sought to compare doses that were closer to physiologic concentration (0.01 and 0.1 μg doses) with those that have been used in previous studies using this experimental model.

Zellin and Linde used recombinant human BMP-2 (rhBMP-2) at doses of 1 μg and 8 μg to heal critical size rat mandibular defects, and found that 80% of defects treated with both doses were bridged with immature bone by 12 days after implantation. By 24 days, 60% of the defects implanted with 1 μg rhBMP-2 and 80% of the defects implanted with 8 μg rhBMP-2 were bridged with new bone, which is in accordance with our results (11). Linde and Hedner compared bone healing in the rat mandibular defect model using 10 μg rhBMP-2 with and without osteopromotive membranes, and found improved bone union in the groups containing rhBMP-2 (12). Similarly, Yoshida et al. demonstrated accelerated bone formation in rat mandibular defects when 10 μg rhBMP-2 was implanted with porous hydroxyapatite beads in a type I collagen carrier (13).

The fact that the smaller doses (0.01 and 0.1 μg) of the growth factors used in this study were not as effective in stimulating new bone growth is due in part to the fact that acute delivery of growth factors was utilized in this study. Exogenously delivered growth factors are turned over rapidly in acute wounds (half-life of a few hours), necessitating the delivery of high doses, which is costly (14). Sustained delivery more closely replicates the normal healing process. Rodent studies of mandibular fracture healing and distraction osteogenesis have shown that BMP-2 through -7 are expressed for periods of weeks after fracture and during consolidation (1,4–6). Sustained delivery has been shown to result in improved bone regeneration when compared with acute delivery and may prove to be more cost-effective because smaller doses of these growth factors delivered over longer periods of time may prove to be as efficacious as larger, acutely delivered doses. Woo et al. compared calvarial defect healing in rabbits using acute and sustained delivery of rhBMP-2 with a biodegradable carrier. In the animals implanted with the sustained-release carrier, 75–79% of the defect area was repaired with new bone growth, when compared with 45% defect healing with the use of the immediate-release implants at equivalent doses (15). In addition, studies have demonstrated that sustained delivery of BMPs results in the formation of coarse, trabecular bone while acute delivery results in the formation of thin, lace-like bone (16,17). The thin bony shells with relatively large marrow spaces that characterized defect healing in this study, particularly with implantation of rhBMP-2, are most likely related to acute growth factor administration.

The relative inefficiency in bone healing seen in this study, especially at the lower doses, may also be related to single dose factor administration. It is recognized that autogenous and allogenic mixtures of BMPs derived from demineralized bone are up to a thousand times more potent for bone induction than any specific recombinant BMP (18). This suggests a potentiating effect of growth factor combinations, and also replicates the natural osseous healing process. In addition, studies indicating that BMP heterodimers demonstrate increased *in vitro* and *in vivo* activities in comparison with respective homodimers corroborate the increased potency of BMP growth factor combinations (19,20).

We were not able to detect significant differences in new bone volumes or area fractions between the growth factors at equivalent doses, although defects implanted with rhBMP-4 tended to have thicker cortical bone and more trabecular bone with interconnectivity. However, at least partial defect bridging was achieved in a greater number of defects implanted with rhBMP-2 (47%) than with rhBMP-4 (35%). Cheng et al. demonstrated a 10-fold greater osteogenic potential of rhBMP-4 in comparison with rhBMP-2 and -7 in their rabbit spinal fusion model by comparing their bony union threshold dose for acute administration of rhBMP-4 (5 μg) with previously reported thresholds for rhBMP-2 and -7 in this model (7). The authors did not comment on the area or volume of new bone generated with rhBMP-4 in comparison with rhBMP-2 or -7. Our inability to demonstrate increased bone healing with rhBMP-4 when compared with rhBMP-2 may be due to differences between membranous and endochondral bone healing. However, like Cheng et al., we were able to demonstrate acceleration of trabecular bone development with rhBMP-4.

Interestingly, 44% of the specimens in the control group implanted with the hyaluronic acid carrier had at least partial defect bridging. We feel that this is due to the osteoconductive nature of these polymers. Hyaluronic acid polymers such as the one used in this have been shown to facilitate bone healing in a rat calvarial defect model (21). Hyaluronic acid gels have also been shown to accelerate bone healing in primate long bone fracture models when applied with basic fibroblast growth factor-2 (22). Like calcium phosphate-based biomaterials, hyaluronic acid-based polymers can

serve as effective scaffolds for marrow-derived mesenchymal stem cells to differentiate into chondrocytes and osteoblasts. In a study comparing two hyaluronic acid polymers to a ceramic calcium phosphate delivery vehicle, the hyaluronic acid sponges were found to bind up to 130% more osteoprogenitor cells than the calcium phosphate vehicle (23). This increased affinity of the mesenchymal cells for the hyaluronic acid vehicles resulted in a 30% increase in bone and cartilage deposition relative to the calcium phosphate carrier. In a canine mandibular alveolar bone-healing model utilizing rhBMP-2, a hyaluronic acid sponge carrier was shown to support complete bone healing, while the use of a collagen carrier resulted in only slight supracrestal bone expansion (24).

Marrow space development occurred in a dose-dependent fashion in this study, and this effect was more pronounced for rhBMP-2. The presence of residual hyaluronic acid implant and fibrous tissue in-growth were inversely correlated with new bone and marrow development as demonstrated in similar previous studies. The effects of sustained delivery of growth factor combinations may prove to be an important area of future study for osseous tissue engineering.

Conclusion

Recombinant human BMP-2 and -4 had similar effects on bone regeneration in rat critical size mandibular defects at equivalent doses. Although administration of rhBMP-4 tended to result in thicker cortical bone and greater trabecular bone areas, a larger percentage of defects implanted with rhBMP-2 had at least partial defect bridging with new bone. The results indicate a threshold dose for acute administration between 1 and 10 μg rhBMP-2 for bony union in this model, and $\geq$10 μg for rhBMP-4. Marrow space development occurred in a dose-dependent fashion in this study, and this effect was more pronounced for rhBMP-2 at larger doses. These findings indicate differences in osteogenic potential between rhBMP-2 and -4 that may have an impact on the translation of osteoinductive protein therapy to clinical practice.

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