In Vitro Effects of Combined and Sequential Bone Morphogenetic Protein Administration

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Objective: To assess the effects of combined and sequential administration of bone morphogenetic protein 2 (BMP-2) and BMP-7 on osteoblastic differentiation compared with administration of single growth factors.

Design: In vitro study of osseous differentiation in murine pluripotent cells using assays of extracellular matrix calcification, alkaline phosphatase activity, and expression of osseous markers. Mesenchymal cells were cultured with BMP-2, BMP-7, or a combination of these growth factors or were sequentially exposed to the growth factors.

Results: Sequential administration of BMP-2 and BMP-7 resulted in increased extracellular matrix calcification and expression of osteocalcin, whereas all groups treated with BMP up-regulated expression of the osteoblastic transcription factor Runx2/cbfa1, type I collagen, and the inhibitory BMP second messenger Smad6. None of the experimental groups demonstrated increased expression of osteopontin or Smad1, and only cells treated with concurrent administration of BMP-2 and BMP-7 increased Smad5 expression. Alkaline phosphatase activity was increased from baseline only in cells treated with BMP-2 alone.

Conclusions: Culture with BMP-2 and BMP-7, their sequential administration, and their coadministration had variable effects on osseous differentiation in mesenchymal cells. These results demonstrate the need for increased understanding of the role of growth factors and their combinations in bone development and have important implications for the ongoing development of osteoinductive therapies.

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growth factors differ in their effects and may demonstrate synergistic or inhibitory effects on bone growth in combination, the interactions of these biomolecules deserve investigation.4-9,11,12

Using an in vitro model with murine mesenchymal cells, the effects of combined and sequential administration of bone morphogenetic protein 2 (BMP-2) and BMP-7 on osteoblastic differentiation was compared with administration of single growth factors. Assays of extracellular matrix calcification, alkaline phosphatase activity, and expression of osseous markers were used to demonstrate osseous differentiation.

## METHODS

### TISSUE CULTURE

The C3H/10T1/2 pluripotent murine mesenchymal cells and 7F2 murine osteoblastic cells were obtained from the American Type Culture Collection (Manassas, Virginia). The 7F2 cells were used as a positive control for osseous differentiation. Both cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 10 U/mL of penicillin, and 0.1 mg/mL of streptomycin at 37°C in a 5% carbon dioxide atmosphere. Both C3H/10T1/2 cells (passage 18) and 7F2 cells were plated into 24-well plates at a density of 2.5 × 10^4 cells per well on day 0. For C3H/10T1/2 cells, the cells were divided into experimental groups as indicated in Table 1, and the medium was changed to a BMP-containing medium with a total of 250 ng/mL of BMP on days 1 and 8 (Table 1). Recombinant human BMP-2 and BMP-7 were obtained from R&D Systems Inc (Minneapolis, Minnesota). The negative control consisted of C3H/10T1/2 cells cultured in the medium without BMPs. On day 1, the medium over the 7F2 cells was also changed to an osteogenic medium that consisted of a culture medium supplemented with 50 µg/mL of ascorbic acid and 10mM β-glycerophosphate (Sigma Chemical Company, St Louis, Missouri). The osteogenic medium over the 7F2 cells was then changed every other day.

### ALIZARIN RED ASSAYS

Calcification of the extracellular matrix was assessed with alizarin red staining. On day 15, the cells were rinsed with distilled water and fixed with 50% ethanol for 15 minutes at 4°C. The fixed cells were rinsed with distilled water and incubated with 1% alizarin red with 0.1% ammonium hydroxide for 45 minutes at room temperature. Excess alizarin red stain was drained, and the wells were rinsed with tap water until clear. The plates were allowed to air dry, and photomicrographs were taken using a 2× magnification objective. A microscope with a color digital camera was interfaced to a computer and used to project images onto a 15-in monitor. Mineralized colonies were counted for each well. Alizarin red assays were performed with 12 replicates for each experimental group.

### ALKALINE PHOSPHATASE ASSAYS

Alkaline phosphatase activity was measured as an indicator of osseous differentiation. On day 15, the medium over the cells in the 24-well plates was aspirated, and the cells were washed twice with phosphate-buffered saline (PBS; Sigma). The cells were then scraped in ice-cold, high-salt buffer (2M sodium chloride and 50mM monobasic sodium phosphate) according to the method of Raiche and Pulco6 and homogenized on ice for 30 seconds. Fifty microliters of each homogenate was combined with 50 µL of 0.67M p-nitrophenyl phosphate in 3 mL of 1M diethanolamine buffer with 0.05mM magnesium chloride (pH, 9.8). After incubation for 30 minutes at room temperature, the reaction was stopped by the addition of 2 mL of 0.05M sodium hydroxide. The concentration of p-nitrophenyl was read by absorption at 410 nm using a SpectraMax 190 microplate spectrophotometer interfaced to a computer with SoftMax Pro software (Molecular Devices, Sunnyvale, California). Alkaline phosphatase assays were performed with 12 replicates for each experimental group, and alkaline phosphatase activity was normalized for DNA content.

### DNA ASSAYS

Cellular DNA content was determined by combining 400 µL of cellular homogenate (see “Alkaline Phosphatase Assays” section) with 400 µL of 200 ng/mL of bisbenzimidezole (Hoechst 33258) in TNE buffer (25 mM Tris hydrochloride, 150 mM NaCl, and 5 mM EDTA) (Sigma). Fluorescence was measured at an excitation wavelength of 358 nm and an emission wavelength of 458 nm using a SpectraMax M5 fluorosecent microplate reader (Molecular Devices) interfaced to a computer with SoftMax Pro software. Standard curves were generated using call thymus DNA diluted in TE buffer (10mM Tris hydrochloride and 1mM EDTA) (Sigma). DNA assays were performed with 12 replicates for each experimental group.

### IMMUNOCYTOCHEMICAL TESTING

Immunocytochemical staining for the osseous markers cbfa1, type I collagen, osteocalcin, osteopontin, Smad1, Smad3, and Smad6 was performed to further assess osseous differentiation of the mesenchymal cells. On day 15, the medium was aspirated from the cells and the cells were rinsed with PBS. The cells were fixed with ice-cold methanol (Fisher Scientific, Hampton, New Hampshire) for 20 minutes at −20°C. The cells were washed twice in PBS, 5 minutes per wash. The cells were then permeabilized for 15 minutes at room temperature with 1% Triton X-100 (Fisher Scientific). The cells were again washed twice. The cells were then blocked with blocking solution consisting of 1% normal serum from the species in which the secondary antibody was made in 0.1% Triton X-100 in PBS with 1% bovine serum albumin (Sigma) for 30 minutes at 37°C. After removal of the blocking solution, cells were incubated with a primary antibody (goat antiosteopontin [2 µg/mL] or anti-cbfa1 [5 µg/mL; R&D Systems]; rabbit anti–type I collagen [1.60 dilution; Calbiochem, San Diego, California]; anti-Smad1 [2 µg/
mL], anti-Smad5 [2 μg/mL], or anti-Smad6 [2 μg/mL; Zymed Laboratories, South San Francisco, California]; or goat antiosteocalcin [1:200 dilution; Biomedical Technologies Inc, Stoughton, Massachusetts]) diluted in PBS with 1% bovine serum albumin for 1 hour at 37°C. The antibody dilutions used were tested in our laboratory using 7F2 cells and were found to result in adequate staining with minimal background using the horseradish peroxidase–diaminobenzidine system (Vector Laboratories, Burlingame, California). Cells were washed 3 times in PBS and incubated with the secondary antibody (biotinylated anti–rabbit or biotinylated anti–goat, 2 μg/mL; Vector Laboratories) for 1 hour at 37°C. Cells were washed 3 times in PBS and incubated with 3 μg/mL of fluorescein-avidin conjugate (Vector Laboratories) in bicarbonate-buffered isotonic sodium chloride solution for 1 hour at room temperature. The cells were again washed twice in PBS. Fluorescence was measured for each well in a fluorescent microplate reader at an excitation wavelength of 495 nm and an emission wavelength of 521 nm. Immunocytochemical assays were performed with 6 replicates for each experimental group.

STATISTICAL ANALYSIS
Data analysis was performed with Statview statistical software (SAS Institute Inc, Cary, North Carolina). One-way analyses of variance were used to identify differences in mean values for alkaline phosphatase activity (expressed as units per microgram of DNA), DNA content (expressed as micrograms per well), and expression of cbfa1, type I collagen, Smad1, Smad5, Smad6, osteocalcin, and osteopontin (expressed as fluorescent units per well). P < .05 was considered statistically significant for all comparisons. The Bonferroni post hoc multiple analysis test was used to identify significant pairwise differences. The importance of significant differences (effect size) was estimated using a Hayes ω² statistic. The effect size indicates the percentage of the total variances that is explained by the independent variable. A ω² of 0.01 through 0.05 indicates a small effect, 0.06 through 0.13 indicates a medium effect, and 0.14 or greater indicates a large effect.

RESULTS

ALIZARIN RED STAINING
Alizarin red staining of 7F2 cells cultivated in osteogenic medium demonstrated large, confluent colonies that could not be counted individually (Figure). In contrast, calcified C3H/10T1/2 colonies were widely separated. Significantly more alizarin red–stained colonies were found in the groups treated with sequential administration of BMP (groups 6 and 7) than in the control group, the groups treated with either single growth factor (groups 1 and 2), or the cells treated with coadministration of BMP-2 and BMP-7 (Table 2).

DNA CONTENT AND ALKALINE PHOSPHATASE ACTIVITY
The DNA content did not differ among any of the experimental groups, indicating that the growth factors had no effect on cellular replication. Alkaline phosphatase activity in the 7F2 osteosarcoma cells exceeded that of all the C3H/10T1/2 groups (P < .001, ω² = 0.21). Among the C3H/10T1/2 groups, alkaline phosphatase activity in group 1 was greater than that in the other experimental groups and the negative control (Table 2).

EXPRESSION OF OSSEOUS MARKERS
Exposure of the murine mesenchymal cells to BMP resulted in increased expression of the osteoblastic transcription factor cbfa1. All experimental groups (except group 2) and the 7F2 osteoblastic cell line demonstrated significantly higher expression of cbfa1 than the C3H/10T1/2 control. In fact, cells in groups 1, 3, and 6 expressed greater levels of cbfa1 than the 7F2-positive control.

Type I collagen expression was greater in all of the C3H/10T1/2 cells, regardless of whether they were exposed to BMP, than in the 7F2 cells. This finding may reflect constitutive expression of type I collagen by this cell line. However, treatment with BMP significantly up-regulated expression of this extracellular matrix protein.

Osteocalcin expression was significantly enhanced only in those experimental groups treated with some combination of BMP-2 and BMP-7 (groups 3 through 7) and not in the groups treated with either single growth factor (groups 1 and 2). Osteocalcin expression in groups 4, 5, and 6 was also significantly greater than its expression by the 7F2 cell line. Conversely, none of the C3H/10T1/2 experimental groups or the negative control expressed significant levels of osteopontin.

Similarly, expression of Smad1 was greater in the 7F2 cells than in the C3H/10T1/2 experimental groups and the negative control. However, expression of Smad5 was comparable between the 7F2 and C3H/10T1/2 cells, regardless of whether the C3H/10T1/2 cells had been treated with BMP. The Smad5 expression was slightly up-regulated only in group 3 cells. Like the expression of type I collagen, Smad6 expression was greater in the C3H/10T1/2 experimental groups and the negative control than in the 7F2 cells. This expression was enhanced by exposure of the C3H/10T1/2 cells to BMP.

COMMENT
Animal studies6,13-16 demonstrate that bone growth factor concentrations and expression of their receptors peak at different times during the healing process. In a rodent model of mandibular fracture healing, Spector et al13 demonstrated intense immunostaining for BMP-7 during the first 2 weeks after fracture. Initially, there was lesser expression of BMP-2 and BMP-4, but the intensity of BMP-2 and BMP-4 staining increased during the next 3 weeks. Staining intensities for all 3 growth factors decreased steadily after the fourth week and returned to baseline by the eighth week. Several studies14,16 have demonstrated strong expression of BMP-2 and BMP-4 and weak expression of BMP-7 during distraction osteogenesis in the rabbit mandibular model. Yazawa et al18 also demonstrated the expression of BMP-5 and BMP-6 throughout the distraction process in their study. These studies17 indicate that growth factors are coexpressed in the mandible during bone regeneration just as they are in the limbs during embryogenesis.
Recombinant human BMP administration in several in vitro and animal models was previously shown to be effective for osteogenesis in many published studies, which is thought to be because the BMPs are highly conserved among species. Approximately 40 BMP isoforms have been identified, and they differ in their effects, which may be mitogenic, chemotactic, morphogenic, or apoptotic depending on the cell type to which the growth factor is exposed and the growth factor concentration. The C3H/10T1/2 pluripotent mesenchymal cell line has been used extensively for studies of osseous and chondral differentiation induced by BMPs. Because they are not transformed, C3H/10T1/2 cells have many similarities to preosteoblastic cell lines, such as the MC3T3-E1 line, which is also used for in vitro osseous differentiation assays. The C3H/10T1/2 cells, however, can exhibit multiple phenotypes (osteoblastic, chondrocytic, and adipocytic) depending on the culture conditions. Although limitations with use of the transformed 7F2 osteoblastic cell line as a positive control are acknowledged, the purpose of this preliminary study was not to compare osseous differentiation between the 2 cell lines but to assess the effects of coadministration and sequential administration of BMP-2 and BMP-7 on undifferentiated cells.

We were able to demonstrate increased extracellular matrix calcification and osteocalcin expression with sequential administration of BMP-2 and BMP-7, although this may have been limited by the dose of BMP used. When administered at bolus doses between 100 and 1000 ng/mL, BMP-2 is able to induce osteoblastic differentiation in C3H10T1/2 cells, and its osteoinductive activity is dose dependent. The concentrations of bone growth factors present during natural, in vivo bone healing are not known. However, naturally occurring BMPs make up 0.1% of total bone weight, indicating that they exist in concentrations of approximately several micrograms per kilogram of bone. Lee et al were able to detect increased expression of alkaline phosphatase and osteocalcin messenger RNA.
within 3 to 7 days after culturing C2C12 myoblasts with BMP-2. Increased osteopontin messenger RNA expression was detected between 3 and 6 days after exposure to BMP-2, and type I collagen messenger RNA up-regulation was detected on day 7. Similarly, Raiche and Puleo\(^6\) were able to detect increased alkaline phosphatase activity in mouse mesenchymal cells after 6 days of culture in BMP-2. We were able to detect increased alkaline phosphatase activity only in the C3H10T1/2 cells treated with BMP-2 alone (group 1). It is possible that BMP-2 and BMP-7 have differential effects on alkaline phosphatase activity in this cell line.

Treatment with both BMP-2 and BMP-7 in this study resulted in increased expression of the Runx2/Cbfa1 osteoblastic transcription factor in the murine mesenchymal cells. Up-regulation of osteocalcin expression in this study mirrored matrix calcification as assessed by alizarin red staining, as would be expected. However, it is recognized that alizarin red staining is a nonspecific marker of matrix calcification and that verification of formation of mineralized bone matrix requires von Kossa staining, electron microscopy, or x-ray diffraction to identify the crystalline structure. Type I collagen expression was increased in all groups treated with BMP but, unlike Lee et al,\(^6\) we did not detect up-regulation of osteopontin in our experimental groups.

Smad proteins are intracellular signal-transducing second messengers for BMPs and other members of the transforming growth factor \(\beta\) superfamily of polypeptide growth factors. Smad1 and Smad5 initiate the transcription of osteoblast-specific genes through activation of Runx2/Cbfa1. Although none of the experimental groups demonstrated up-regulation of Smad1 expression in response to BMP exposure, Smad5 expression was equal to that of 7F2 osteoblastic cells, indicating that Smad5 may be constitutively expressed by the C3H10T1/2 mesenchymal cells. Interestingly, the expression of Smad6, which inhibits BMP signal transduction, was greater in the C3H10T1/2 cells. Although some markers of osseous differentiation, such as increased matrix calcification and up-regulation of Runx2/Cbfa1 and osteocalcin expression, were enhanced by coadministration and/or sequential administration of BMP-2 and BMP-7 to the murine mesenchymal cells, sequential administration of BMPs may not necessarily improve in vivo bone regeneration. Certainly, in vitro studies using cultured osteoblasts, as well as in vivo studies, are needed to assess the efficacy of sequential BMP administration in osteoblastic differentiation compared with administration of single growth factors. The order of BMP administration did not seem to have bearing on increased matrix calcification or up-regulation of Cbfa1.

In conclusion, culture with BMP-2 and BMP-7 individually, their coadministration, and their sequential administration had differential effects on osteogenic differentiation in the C3H10T1/2 murine mesenchymal cell line. Although these effects may partly reflect the unique physiologic features of this cell line, the enhanced matrix calcification and expression of osseous markers demonstrated with sequential administration of BMP-2 and BMP-7 may reflect a more biomimetic administration of these growth factors for bone regeneration. These results demonstrate the need for increased understanding of the role of growth factors and their combinations in bone development and have important implications for the ongoing development of osteoinductive therapies.

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Author Contributions: Dr Arosarena had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Arosarena. Acquisition of data:

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Table 2. Alizarin Red Staining, DNA Content, and Osteoblastic Marker Expression for Each Experimental Group

<table>
<thead>
<tr>
<th>Group</th>
<th>Alizarin Red-Stained Colonies, No. per Well</th>
<th>Alkaline Phosphatase Activity, U/jug of DNA</th>
<th>DNA, (\mu g) per Well</th>
<th>Cbfa1, U per Well</th>
<th>Type I Collagen, U per Well</th>
<th>Osteocalcin, U per Well</th>
<th>Osteopontin, U per Well</th>
<th>Smad1, U per Well</th>
<th>Smad5, U per Well</th>
<th>Smad6, U per Well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control group</td>
<td>3.17 ± 3.12</td>
<td>0.06 ± 0.07</td>
<td>1035.28 ± 247.14</td>
<td>7.29 ± 1.88</td>
<td>4.80 ± 1.60</td>
<td>3.74 ± 0.55</td>
<td>1.11 ± 0.05</td>
<td>1.06 ± 0.08</td>
<td>11.23 ± 2.55</td>
<td>10.42 ± 0.78</td>
</tr>
<tr>
<td>Positive control group</td>
<td>NA</td>
<td>1.24 ± 1.74</td>
<td>933.27 ± 373.78</td>
<td>13.03 ± 2.49</td>
<td>3.20 ± 0.42</td>
<td>10.56 ± 2.10</td>
<td>12.01 ± 2.36</td>
<td>2.85 ± 0.49</td>
<td>12.01 ± 2.36</td>
<td>2.21 ± 0.15</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not applicable.

Data are presented as mean ± SD.
Arosarena. Analysis and interpretation of data: Arosarena and Puleo. Drafting of the manuscript: Arosarena. Critical revision of the manuscript for important intellectual content: Arosarena and Puleo. Statistical analysis: Arosarena. Obtained funding: Arosarena. Administrative, technical, and material support: Arosarena and Puleo. Study supervision: Arosarena.

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