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Combinatorial Gene Therapy with BMP2/7 Enhances Cranial Bone Regeneration

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Abstract

BMP2/7 heterodimer expression by adenovirus can stimulate bone formation at subcutaneous sites. In the present study, we evaluate whether this approach will also promote healing of cranial defects. Adenovirus expressing BMP2 or BMP7 (AdBMP2, AdBMP7) was titrated to yield equivalent BMP protein levels after transduction into murine BLK cells. Analysis of conditioned medium showed that BMP2/7 heterodimers have enhanced ability to stimulate alkaline phosphatase and Smad 1,5,8 phosphorylation relative to equivalent amounts of BMP2 or BMP7 homodimers. To measure bone regeneration, we implanted virally transduced BLK cells into critical-sized calvarial defects generated in C57BL6 mice. AdBMP2/7-transduced cells were more effective in healing cranial defects than were cells individually transduced with AdBMP2 or BMP7. Dramatic increases in bone volume fraction, as measured by microCT, as well as fusion of regenerated bone with the defect margins were noted. Thus, the use of gene therapy to express heterodimeric BMPs is a promising potential therapy for healing craniofacial bones.

Keywords

gene therapy; bone regeneration; adenovirus; bone morphogenetic proteins; heterodimer; cranial defect

INTRODUCTION

Bone morphogenetic proteins (BMPs) induce endochondral bone formation by stimulating the differentiation of mesenchymal progenitor cells (Hogan, 1996). Recombinant BMPs have been widely evaluated in several applications, including treatment of spinal fusions, non-union fractures, craniomaxillofacial and periodontal bone defects, and bone/tooth implant augmentation (King *et al.*, 1997; Giannobile *et al.*, 1998; Geesink *et al.*, 1999; Cochran *et al.*, 2000; Kirker-Head, 2000; Franceschi, 2005). Although in many cases the efficacy of BMP protein therapy has been demonstrated, problems with protein stability at the implant site,

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inadequate understanding of optimal timing and conditions for BMP delivery, and cost have all limited the use of BMPs in the clinic.

More recently, gene therapy has been explored as an alternative to protein therapy. With this approach, it is possible to achieve sustained BMP expression at regeneration sites and, in many cases, bone regeneration that is superior to that achieved with recombinant proteins (Lieberman *et al.*, 1998, 1999; Riew *et al.*, 1998; Krebsbach *et al.*, 2000; Winn *et al.*, 2000; Govender *et al.*, 2002; Rutherford *et al.*, 2002; Franceschi *et al.*, 2004). With gene therapy, it has also been possible to express combinations of regenerative molecules simultaneously, including multiple BMP subtypes, angiogenic factors such as VEGF, or the osteogenic transcription factor, Runx2 (Peng *et al.*, 2002; Zhu *et al.*, 2004; Huang *et al.*, 2005; M Zhao *et al.*, 2005; Z Zhao *et al.*, 2005, 2007). Previously, we used adenovirus-based gene therapy to express unique combinations of BMPs 2, 4, and 7 and found that the BMP2/7 combination had enhanced osteogenic activity (M Zhao *et al.*, 2005). This result was explained by the formation of BMP2/7 heterodimers having substantially greater biological activity than individual homodimeric BMPs. A similar strategy was also previously used to stimulate spinal fusion in rats (Zhu *et al.*, 2004).

In the present study, we further explore the therapeutic potential of combinatorial gene therapy by assessing the ability of AdBMP2/7 to heal a calvarial critical-sized defect. We also compare the ability of homo- and heterodimeric BMPs to stimulate known kinase pathways associated with BMP signal transduction.

MATERIALS & METHODS

Adenovirus Construction

Recombinant adenoviruses (Ad) encoding BMP2 and BMP7 were constructed by Cre/lox recombination (M Zhao *et al.*, 2005). Purified viruses were titered and stored in 20% glycerol-phosphate-buffered saline. AdLacZ, which encodes bacterial β -galactosidase, was used as a vector control.

Cell Culture Studies

Mouse myoblast (C2C12 cells) and fibroblast (BLK cells) lines were used in this study. For adenovirus transduction and preparation of conditioned medium (CM), BLK cells were plated at a density of 75,000 cells/cm² in DMEM plus 2% FBS. After 24 hrs, culture medium was changed to serum-free medium, and CM was collected after an additional 24 hrs. BMP 2 and 7 protein levels in CM were determined by ELISA (R&D Systems, Inc., Minneapolis, MN, USA). For measurement of alkaline phosphatase (ALP), C2C12 cells were cultured for 4 days with BMP-containing CM. Cell lysates were prepared and assayed for alkaline phosphatase (ALP) (Manolagas *et al.*, 1981) and DNA by means of a Picogreen[®] dsDNA quantitation kit (Molecular Probes, Inc., Eugene, OR, USA). Smad and p38 MAPK signaling was analyzed on Western blots as previously described (Phimphilai *et al.*, 2006).

Animal Experiments

All procedures were approved by the University Committee on the Use and Care of Animals and were in compliance with State and Federal laws. BLK cells were infected with AdLacZ, AdBMP2, or AdBMP7, alone or in combination. Viral titers were adjusted such that cells secreted an equivalent amount of each BMP. The total virus titer was held constant at a multiplicity of infection (moi) of 210 by the addition of the appropriate amounts of AdLacZ. Twenty-four hrs after virus treatment, cells were trypsinized and adsorbed to a gelatin sponge (Gelfoam; Upjohn, Kalamazoo, MI, USA) (Krebsbach *et al.*, 2000). Four million cells were used in each implant (6 implants/group). Surgical procedures were performed on four- to five-

week-old C57BL/6 mice (Charles River Laboratories, Inc., Wilmington, MA, USA), as described previously (Koh *et al.*, 2006). Briefly, mice were anesthetized, a 0.8- to 1.0-cm sagittal incision was made on the scalp, and the calvarium was exposed by blunt dissection. A critical-sized defect was created by means of a 7-mm-diameter trephine bur (Fine Science Tools, Foster City, CA, USA). A pre-formed gelatin sponge adsorbed with AdBMP-treated BLK cells was placed in the defect, and the incision was closed with 4-0 absorbable chromic gut (Ethicon Inc., Somerville, NJ, USA). Cranial bones were harvested 4 wks after implantation.

Microradiography, μ CT Analysis, and Histology

A Faxitron microradiographic apparatus (Faxitron X-ray Corp., Wheeling, IL, USA) was used for the visualization of *in vivo* bone regeneration. Isolated cranial bones were exposed to x-ray film at 35 kV for 15 sec. Samples were then fixed in 10% formaldehyde/PBS overnight and stored in 70% ethanol. For three-dimensional analysis by μ CT, samples were scanned by a model MS8-CMR-100 scanner (EVS Corp, London, ON, Canada) at 10- μ m voxel resolution and 80 kV. Three-dimensional isosurface renderings were made with MicroView v 1.1.1 (GE Medical Systems, London, ON, Canada) for the visualization of bone. For histology, isolated cranial bones were fixed in 10% formaldehyde for 1 day, and then demineralized with 10% EDTA for 1 wk at 4°C before being embedded in paraffin and sectioned at 8 μ m. Slides of sections were stained with hematoxylin and eosin.

Statistical Analysis

Experimental values are reported as means $\pm$ SD. Statistical significance was assessed by a Tukey-Kramer multiple-comparisons test with an Instat 4.0 software package.

RESULTS

Conditioned Medium from AdBMP2/7- transduced Cells Synergistically Stimulates Osteoblast Differentiation in C2C12 Cells

We previously showed that co-transduction of AdBMP2 and AdBMP7 into a variety of mesenchymal cell lines synergistically stimulated osteoblast differentiation and gene expression relative to cells individually transduced with AdBMP2 or 7 (M Zhao *et al.*, 2005). These results were explained by the formation of BMP2/7 heterodimers that were detected by functional assays and immunoprecipitation when both BMPs were expressed in the same cell. For a more detailed comparison of activities of homo- and heterodimeric BMPs, conditioned medium (CM) was prepared from BLK fibroblasts transduced with control AdLacZ adenovirus, AdBMP2, AdBMP7, or the AdBMP2/7 combination. Individual viral titers were adjusted to give similar levels of all 3 BMPs as measured by ELISA of CM collected 24 hrs after transduction (approx. 150 ng of each BMP/mL, Fig. 1A). Because the predicted molecular weights of all 3 BMP species are similar (between 28 and 32 kD), this is equivalent to a concentration of approximately 547 nM. We then compared the ability of each CM to stimulate alkaline phosphatase activity dose-dependently when added to C2C12 cells (Fig. 1B). The enhanced ability of BMP2/7 to stimulate this osteoblast marker is clearly evident, particularly at the lowest dilutions, where BMP2 or BMP7 was inactive. Also note that mixing AdBMP2 with AdBMP7 CM (BMP2+7 group) did not give the synergistic stimulation seen in cells transduced with the AdBMP2/7 combination, even though equivalent amounts of each BMP were present. This is consistent with the presence of BMP2/7 heterodimers in the AdBMP2/7 CM that cannot form when AdBMP2 and AdBMP7 CM are mixed (M Zhao *et al.*, 2005).

Synergistic Stimulation of Smad Phosphorylation by BMP2/7-containing Conditioned Medium

BMPs signal by binding to types I and II BMP receptors. The resulting receptor activation initiates two types of signals, the canonical SMAD pathway and mitogen-activated protein kinase (MAPK) pathways. BMP receptor activation stimulates phosphorylation of SMADs 1, 5, and 8 that subsequently dimerize with the common partner protein, SMAD4, and translocate to the nucleus to regulate the transcription of target genes (Nohe *et al.*, 2004). Depending on the cell system examined, BMPs also activate several mitogen-activated protein kinases (MAPKs), including ERK, p38, and JNK (Nakamura *et al.*, 1999; Hassel *et al.*, 2003; Nohe *et al.*, 2004). To begin examining the mechanistic basis for the enhanced activity of BMP2/7 heterodimers, we compared the ability of BMP2 homodimer, BMP7 homodimer, or BMP2/7 heterodimers to stimulate SMAD, p38, ERK, and JNK pathways in C2C12 cells (Fig. 1C; only results for SMAD and p38 pathways are shown). BMP2 CM and, to a much lesser extent, BMP7 increased SMAD1/5/8 phosphorylation without altering total SMAD levels (Fig. 1C, top). The addition of equivalent amounts of BMP2 plus BMP7 CM also stimulated SMAD phosphorylation, but no more than was seen with BMP2 alone. In contrast, CM containing BMP2/7 heterodimers was much more active than any of the other groups, consistent with it also having increased activity in stimulating osteoblast differentiation. No single BMP or BMP combination affected p38, ERK, or JNK phosphorylation, indicating that these pathways do not play a major role in BMP signal transduction in C2C12 cells. In conclusion, the canonical SMAD pathway can mediate effects of heterodimeric BMPs.

Combinatorial Gene Therapy with AdBMP2/7 Shows Enhanced Ability to Repair a Critical-sized Cranial Defect

Using a subcutaneous implant model, we previously showed that cells transduced with the AdBMP2/7 combination produced more ectopic bone than those individually expressing BMP 2 or 7 (M Zhao *et al.*, 2005). However, that study did not evaluate osteogenic activity in a clinically relevant bone defect model. In the present study, we used a calvarial defect to assess the possible therapeutic value of using gene therapy to express BMP2/7 heterodimers. A 7-mm defect was created in the calvarial bones of C57BL6 mice. This critical-size defect cannot spontaneously heal during a four-week period (Koh *et al.*, 2006). BLK fibroblasts were transduced with AdLacZ, AdBMP2, AdBMP7, or the AdBMP2/7 combination under the same conditions (Fig. 1) (all cells secreted approx. 150 ng BMPs/24 hrs). BLK cells are derived from C57BL6 and can be implanted into this mouse strain (M Zhao *et al.*, 2005). Cells (4×10^6 /implant) were adsorbed to a collagen carrier and implanted into defects. After 4 wks, cranial bones were harvested and analyzed by Faxitron x-ray (Fig. 2), microcomputed tomography (microCT, Fig. 3), and histology (Fig. 4). AdLacZ-transduced cells formed minimal amounts of new bone. However, AdBMP2 or AdBMP7 both induced partial healing, with the activity of AdBMP2 being somewhat higher than that of AdBMP7. In contrast, the AdBMP2/7 combination exhibited robust osteogenic activity, with complete coverage of defects in most samples. We used microCT scans to calculate new bone area with each treatment (6 samples/group), compared with intact control calvaria. Bone volumes expressed as a percentage of control calvaria were as follows: AdLacZ, 6.5 ± 1.2 ; AdBMP2, 49 ± 10.7 ; AdBMP7, 40.5 ± 3.8 ; and AdBMP2/7, 82.0 ± 17.0 . Bone area in all AdBMP-treated groups was increased when compared with the AdLacZ vector control ($p < 0.001$). Furthermore, the AdBMP2/7 group formed significantly more bone than either of the individual BMPs ($p < 0.01$). The extensive bone formation in the AdBMP2/7 group is also apparent in histological sections (Fig. 4). Unlike AdBMP2- or AdBMP7-treated groups, new bone was continuous with margins of the defect (arrowheads). However, the robust osteogenic activity of the AdBMP2/7 vector combination also caused considerable overgrowth of new bone, leading to a disorganized structure apparent in both histological sections and microCT views of defects.

DISCUSSION

In this study, we show that combinatorial gene therapy with adenovirus vectors to express BMP2/7 heterodimers can enhance bone regeneration in a craniofacial defect when compared with vectors individually expressing homodimeric BMPs 2 and 7. This approach restored bone volume to greater than 80% of levels seen in intact calvarial bones, and new bone appeared to be continuous with the defect margins. This work shows that the use of gene therapy to express heterodimeric BMPs may be useful for the therapeutic repair of craniofacial bones.

One complicating factor was that the AdBMP2/7 combination caused overgrowth around the calvarial defect. This was likely a consequence of the high levels of heterodimeric BMPs produced by adenoviruses containing the strong cytomegalovirus promoter. To address this issue, we could treat defects with lower titers of AdBMP2/7 or reduced numbers of transduced cells, or a regulated expression system could be used to control the level and duration of BMP synthesis. As shown, CM from AdBMP2/7-transduced BLK cells dose-dependently stimulated osteoblast differentiation, and had biological effects, even at low concentrations. Thus, it may be possible to limit bone overgrowth by lowering viral titer or the number of implanted cells. An additional advantage of lowering titers is that possible toxicity and/or immune reaction to virus might be minimized. We recently developed a rapamycin-regulated BMP2 expression system that could be adapted for regulated expression of BMP2/7 (Koh *et al.*, 2006).

Although it has been known for many years that heterodimeric BMPs have increased biological activity (Aono *et al.*, 1995), there is currently no mechanism to explain this observation. Besides Smads, BMP receptors can also stimulate non-Smad signaling, resulting in activation of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases p42/p44 (ERK1/2), and c-Jun N-terminal kinase (JNK) (Nakamura *et al.*, 1999; Hassel *et al.*, 2003; Nohe *et al.*, 2004). In the present study, BMP2/7 heterodimers preferentially stimulated Smad 1,5, and 8 phosphorylation without affecting MAPK pathways. Thus, heterodimeric BMPs can elicit at least some of their effects *via* canonical Smad signals, although MAPK signaling may participate in this response in certain cell types.

There are at least two possible ways BMP2/7 heterodimers might preferentially stimulate BMP receptor activation: First, they may induce formation of unique tetrameric receptors with enhanced signaling properties. Dimeric BMPs induce clustering of tetrameric receptors containing two type I and two type II receptors. BMP2 preferentially binds and activates the type I receptors, activin receptor-like kinase 3 (ALK-3 or BMPR1A) and ALK-6 (BMPR1B), while BMP7 preferentially binds to ActR-IA (ALK-2) (ten Dijke *et al.*, 1994; Yamashita *et al.*, 1995; Nishitoh *et al.*, 1996). Therefore, BMP 2/7 heterodimers may induce the formation of unique tetrameric species by combining the binding properties of BMP2 and BMP7 to activate three type I receptors, ALK-3, ALK-6, and ALK-2, simultaneously. Consistent with this model, in the present study, BMP2/7 heterodimers stimulated Smad1/5/8 phosphorylation to a greater extent than was seen with either the BMP2 or the BMP7 homodimer, or a mixture of the two. Also, combined transduction of C2C12 cells with constitutively active ALK-2 and ALK-3/6 was shown to activate BMP signaling and osteoblast differentiation to a much greater extent that was seen with either receptor alone (Aoki *et al.*, 2001). A second possibility is that heterodimeric and homodimer BMPs differ in their ability to regulate the synthesis of BMP inhibitors and/or are differentially affected by these inhibitors. In this regard, BMP2/7 heterodimer was a weaker inducer of the BMP inhibitor, Noggin, than were homodimeric BMPs, and was also resistant to Noggin inhibition (Zhu *et al.*, 2006). These concepts still need to be explored in greater detail by analysis of BMP receptor complexes and binding affinity of heterodimeric BMPs to Noggin and BMP receptors.

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Figure 1.

Biological activity of conditioned medium (CM) from cells transduced with AdBMP2/7. **(A)** BMP content of conditioned medium. BLK fibroblasts were transduced with AdLacZ (vector control), AdBMP2 (moi 150), AdBMP7 (moi 60) alone, or the AdBMP2 plus AdBMP7 combination (same moi). Total viral titer was held constant at 210 moi by addition of the appropriate amount of AdLacZ. CM was collected after 24 hrs. A portion was used for measurement of BMP levels by ELISA, and the remainder was stored at -70°C for functional assays. As shown, for the specific lots of AdBMP2 and 7 used in this study, these viral titers directed the production of equivalent amounts of BMP2 and BMP7 in CM. **(B)** Alkaline phosphatase induction. C2C12 cells were plated in 24-well plates and incubated in a total

volume of 300 μ L containing up to 100 μ L of each CM or combinations of CM. Volumes were equalized by the addition of the appropriate volume of CM from AdLacZ-treated cells. After 5 days, cells were harvested for measurement of ALP activity and DNA. Values are reported as means $\pm$ SD, N = 3. (C) SMAD and p38 phosphorylation. C2C12 cells were treated with BMP-containing CMs as described in (B). Cells were separately harvested at 2 hrs for Smad1/5/8 and at 30 min for p38. Phospho and total SMADs and p38 were measured on Western blots as described in MATERIALS & METHODS.

Figure 2.

Radiographic study of the repair of critical-size cranial defects by AdBMP2/7 combinations. BLK fibroblasts were transduced with each virus or combinations under the conditions described in Fig. 1, and 4×10^6 cells were implanted into 7-mm cranial defects as described in MATERIALS & METHODS. After 4 wks, cranial bones were harvested and analyzed by means of a Faxitron x-ray apparatus. So that readers can appreciate the experimental variations observed, all 6 calvarial samples/experimental group are shown, as well as 4 intact calvaria from control animals.

Figure 3.

Micro-computed tomography analysis of cranial defects. Representative three-dimensional images were rendered from μ CT data. Dorsal (top) and ventral (bottom) three-dimensional renderings of calvarial bones are shown. These images were generated from calvarial samples shown in Fig. 2 (second column from left).

Figure 4. Histology. Coronal sections through the midline of defects are shown. Margins of the original 7.0-mm trephine defect are shown (arrowheads). The same samples as shown in Fig. 3 were used for histology. Bar, 1 mm.