Use of bone morphogenetic protein–9 gene therapy to induce spinal arthrodesis in the rodent


Departments of Neurosurgery, Biomedical Engineering, and Radiology, University of Virginia Medical Center, Charlottesville, Virginia; and Genetics Institute, Cambridge, Massachusetts

Object. Bone morphogenetic proteins (BMPs) have been shown to have significant osteoinductive activity in numerous in vitro and in vivo assay systems, and BMP-2 and BMP-7 are currently being evaluated in human clinical studies. In the spinal region, BMPs have been shown to promote spinal arthrodesis at a higher rate than autologous bone alone. The delivery of BMPs via direct or ex vivo gene therapy techniques is also currently being evaluated and has shown promise in several mammalian models. The present study was designed to evaluate the efficacy of the use of direct, percutaneous BMP-9 adenoviral gene therapy to promote spinal fusion in the rodent.

Methods. Each animal was injected with $7.5 \times 10^8$ pfu of a BMP-9 adenoviral vector in the lumbar paraspinal musculature and allowed to survive 16 weeks. Computerized tomography studies and histological analysis demonstrated massive bone induction at the injection sites, clearly leading to solid spinal arthrodesis, without evidence of pseudarthroses, nerve root compression, or systemic side effects.

Conclusions. The results of this study strongly support the advancement of BMP gene therapy techniques toward clinical use.

Key Words • spinal fusion • bone morphogenetic protein–9 • gene therapy • adenoviral vector • bone morphogenetic protein

Bone morphogens will probably play an important role in the future for the treatment of numerous pathological processes of the spine, including congenital deformity, degenerative and acquired spinal instability, osteoporosis, and neoplasia. Although many growth and differentiation factors have been shown to have osteoinductive activity, several members of the BMP family have demonstrated the highest efficacy in preclinical and clinical studies.7,20,39-41 The BMPs belong to the transforming growth factor–β superfamily based on amino acid homology and, in addition to being involved in skeletal histogenesis, are also intricately involved in the development of the brain, spinal cord, liver, kidneys, skeletal muscle, eyes, and epithelium.8,32,41 However, the osteogenic properties of several of the BMPs have attracted the greatest research interest, and significant efforts are currently underway to advance their use in the clinical setting.31

Human recombinant BMP-2 and BMP-7, when applied on a collagen sponge, have been shown to promote spinal arthrodesis in rodents, rabbits, dogs, and primates.13,15,16,14,20,21,26,28,33-37,42 Importantly, even though these powerful growth factors have been placed in close proximity to nerve roots and the spinal cord, no studies have demonstrated neural compression caused by excessive bone formation.21 Because the preclinical animal experimental results have been so compelling, BMP-2 is now being evaluated in human clinical trials to determine both its safety and efficacy in promoting spinal fusion.

The direct application of rhBMPs, however, is limited by short-term bioavailability, the need for a complex biological carrier, and an inability to control growth factor release over time. In addition, the proteins cannot be given intravenously to induce bone deposition in specific tissues. The expression and secretion of BMPs through gene therapy techniques may overcome some of these limitations and improve the efficacy of BMP therapeutics. Several research groups have recently demonstrated that ex vivo gene therapy techniques may be useful for expressing BMP-2 in ectopic locations, leading to endo-
chondral bone formation. Direct delivery of the BMP-2 gene via a first-generation adenoviral vector has also been shown to promote osteoinduction strongly in the thigh musculature of athymic nude rats. Several research groups have also demonstrated the ability of ex vivo and direct BMP-2 gene therapy to induce spinal fusion in rodents and rabbits. In addition, direct BMP-2 and BMP-9 adenoviral gene therapy has been previously shown to be effective in repairing bony defects in the craniofacial region. It may be possible, however, that other bone morphogens or a cocktail of proteins may have significantly more osteoinductive activity than BMP-2. For example, Boden et al. have recently reported that TIM mineralizing protein-1 ex vivo gene therapy can successfully induce spinal fusion in rodents, apparently by upregulating the expression of a variety of bone morphogens.

Although BMP-9 has been shown to exert only mild in vivo osteoinductive effects when applied to a biological carrier, BMP-9 gene therapy may be more potent and efficacious. A first-generation BMP-9 adenoviral vector has been shown to induce massive amounts of ectopic bone formation in the thigh musculature of immunocompetent and athymic nude rats, and it seems to promote endochondral bone formation at an extremely fast rate (Fig. 1). Because BMP-9 gene therapy may prove to be a more effective technique than BMP-2 gene therapy for promoting ectopic bone formation, the present study was undertaken to determine whether a BMP-9 adenoviral vector injected percutaneously can induce spinal arthrodesis in the athymic nude rodent.

Materials and Methods

The host immune response has been shown to significantly limit transgene expression of first-generation adenoviral vectors. For this reason, eight 16-week-old male athymic nude rats were used for this study.

Adenoviral Constructs

First-generation, Type 5 adenoviral vectors that were made replication defective through complete deletion of the E1a and E1b regions and through partial deletion of the E3 region of the viral genome were used in this study. The Ad-BMP-9 (Genetics Institute, Andover, MA) was constructed with the BMP-9 gene under the control of a cytomegalovirus promoter. The BMP-9 complementary DNA was a chimera of the murine BMP-9 proregion (first two thirds of N, terminus) and the human mature region. A second adenoviral vector containing the β-galactosidase (β-gal) gene under the control of the cytomegalovirus promoter (Ad-β-gal) was used as a control. The viruses were propagated on 293 cells and purified by two rounds of cesium chloride centrifugation. The purified virus was dialyzed in PBS. These viruses were stored until use at −80°C in PBS, 10% glycerol at a concentration of 10^10 pfu/μl.

Injection Procedure

The eight rats were anesthetized with a mixture of ketamine and xylazine, and the lumbosacral area was prepared in a sterile fashion. With fluoroscopic guidance, each animal underwent paraspinal, percutaneous injection at the lumbosacral junction with 7.5 μl (7.5 × 10^10 pfu) of virus with Ad-BMP-9 on the right and Ad-β-gal on the left. To ensure correct placement of the vector, a 19-gauge guide needle was inserted in the junction of the spinous process and lamina on each side. A Hamilton microsyringe was inserted through this needle, and 7.5 μl of the viral solution (7.5 × 10^10 pfu) was injected.

Imaging Analysis

The animals were sedated, and CT scans of the lumbosacral junction were obtained at Weeks 4, 6, 12, and 16 postinjection. Axial CT images with a 1-mm collimation and 1-mm table increment were obtained using the standard algorithm with 130 kV, 100 mA, a 2-second scan time, and a 40-mm image size. The three-dimensional reconstruction was performed using a Voxel Q workstation.

Histological Analysis

At 16 weeks postinjection, the rats were anesthetized and underwent transcardial perfusion with 100 ml of PBS followed by 350 ml of a 4% paraformaldehyde solution. The lumbosacral spines were harvested and treated with a decalcifying solution composed of 10% HCl and 0.1% ethylenediamine tetraacetic acid. The specimens were dehydrated through a series of graded ethanols, xylol, and finally, xylene, after which the spines were infiltrated and embedded in paraffin. Using a microtome, the tissue was sectioned into 16-μm slices and mounted on treated slides. After drying overnight, the slides were stained with Alcian blue (pH 2.5), nuclear fast red, and hematoxylin and eosin, after which all slides were coverslipped.

Results

None of the Ad-BMP-9–injected animals displayed evidence of systemic or local toxicity related to the gene therapy treatment. In addition, the animals remained neurologically intact throughout the postoperative period and no evidence of meningeal irritation or nerve root compression was demonstrated.

Computerized Tomography Studies

Computerized tomography scans demonstrated clear evidence of osteogenesis at the paraspinal Ad-BMP-9 injection sites (Fig. 2 upper left). The newly formed bone appeared to be in direct contact with the host spinous processes and laminae, without evidence of any intervening soft tissue. The volume of induced bone was significant, extending posteriorly to the top of the spinous process and laterally to the facet joints. There was no evidence of spinal canal compromise by the development of bony overgrowth deep to the ligamentum flavum. The volume of new bone formation was subsequently visualized in three-dimensional CT reconstructions (Fig. 2 upper right and lower left and right).

Histological Findings

Histological examination of the injection sites 4 months after treatment demonstrated a well-developed fusion mass consisting of lamellar bone containing normal bone marrow elements (Fig. 3). Because only small amounts of residual cartilaginous tissue were found within the fusion mass, we inferred that the tissue had almost completely progressed through the endochondral bone formation pathway. The lamellar bone contained mature osteocytes, and normal–appearing osteoblasts and osteoclasts lined the marrow cavities. The marrow spaces contained histologically normal bone marrow cells, including lymphocytes and adipocytes. The induced bone was clearly demarcated from the surrounding paraspinal musculature, and there appeared to be no normal or atrophied muscle fibers traversing the fusion mass. In addition, the bone was well integrated with the adjacent host bone, although some areas were found to demonstrate persistent host periosteum, which appeared to have a cartilaginous phe-
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Discussion

In the present study, we have demonstrated that BMP-9 gene therapy has the potential to become a useful, minimally invasive technique to induce spinal arthrodesis. In a previous study, our group clearly demonstrated the ability of Ad-BMP-2 to induce osteogenesis in the paraspinal region, although the volume of bone formed was significantly less than that in the current study. This is consistent with previous rodent BMP-2 and BMP-9 gene therapy studies performed in the thigh musculature. Although a time course study of the histological findings obtained at the spinal BMP-9 injection site was not performed, the results of a previous study clearly demonstrated the mechanisms by which Ad-BMP-9 induces bone formation in ectopic locations (Fig. 1). The Ad-BMP-9 transduces the muscle fibers, which express and secrete BMP-9. The extracellular BMP-9 subsequently stimulates pluripotent stem cells to migrate to the injection site and proliferate between the muscle fibers. These cells then differentiate into small chondrocytes that produce a loose cartilaginous matrix, and the matrix calcifies to form woven bone, which finally remodels into normal lamellar bone.

Song, et al., have demonstrated that BMP-9 receptors appear to be similar in size to the typical Type I and Type II receptors used by transforming growth factor–β family members. Therefore, BMP-9 most likely phosphorylates a Smad second messenger system intracellularly to regulate gene expression in the nucleus. Cells transduced by this vector will express the bone morphogen when the experimental animal is treated with tetracycline or one of its derivatives, but gene expression should drop to nonphysiological levels in the absence of tetracycline. The development of inducible promoters is currently in its early stages, although we anticipate that with increasing interest in gene therapy, novel inducible systems with tighter control of gene expression should be available in the near future.

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However, none of the treated rodents demonstrated evidence of hepatic or local toxicity related to the paraspinal Ad-BMP-9 injections.

The application of BMPs and BMP gene therapy may not be limited to spinal arthrodesis. The morphogens may also play a crucial role in the treatment of spinal fractures and osteoporosis. Direct injection of the BMP vector into the fractured or osteopenic vertebral body may stimulate the recruitment and proliferation of stem cells into the marrow cavity, and have a direct proliferative effect on local osteoblasts, leading to focal bone deposition at the injection site. However, because the bone marrow cavity is essentially a venous channel with a relatively high blood flow rate, the vector may be washed out into the peripheral circulation before adequate cellular transfection has occurred. Hence, larger volumes of virus may need to be applied to these regions. This could lead to increased systemic toxicity and may, therefore, need to be modified by injecting the virus in embolic agents.

To further target expression of the bone morphogen to the treatment site, transduclional or transcriptional targeting may also prove to be useful.27 Transductional targeting techniques involve the surface modification of viral or nonviral vectors with antibodies so that they have a propensity to bind to specific cell types. This approach may increase not only cell specificity but also the transfection rate. In transcripotional targeting, on the other hand, tissue-specific promoters are used to drive the transgene of interest in cell types that normally express the promoter’s gene. For example, the myosin promoter could be used to target transgene expression to muscle cells, whereas the osteocalcin promoter would limit expression to osteoblasts and osteocytes.23,24,27 Using these promoters, gene expression would be limited to the injection site, because leakage of the vector into the peripheral circulation would be cleared by hepatocytes, which would not express the transgene. The combination of a cell specific gene expression with an inducible system is even more compelling, especially for the treatment of diffuse, chronic bone disorders such as osteoporosis. As more bone morphogenic proteins are identified, their specific in vivo activity will need to be assessed in detail to maximize

Fig. 2. Results of CT studies. Upper Left: A CT scan of athymic nude rat spine treated with Ad-BMP-9 in the lumbar region. Note large bony mass (arrows) with a solid interface with the normal adjacent spine and normal spinal canal. The paraspinal muscle treated with Ad-β-gal shows no evidence of bone formation (asterisk). Three-dimensional CT reconstructions at several fusion sites (arrows) are shown in the superior (upper right), posterior (lower left), and lateral views (lower right).
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Fig. 3. Light photomicrograph of an axial view of a paraspinal Ad-BMP-9 injection site, demonstrating large fusion mass (arrows) integrating well with the adjacent lamina and spinous process. Note normal-appearing nerve roots (arrowheads) in the spinal canal. The side treated with Ad-β-gal shows no evidence of bone formation (asterisk). Alcian blue, nuclear fast red, and H & E, original magnification × 30.

their usefulness in the clinical setting. For example, some bone morphogens may induce a strong chemotactic response, whereas others may have a stronger effect on osteoblast proliferation and osteoid deposition. Eventually, a cocktail of vectors expressing different homodimeric and/or heterodimeric BMPs in a specific temporal fashion will maximize the volume of newly formed bone, minimize the time course for osteoinduction, and improve the final biomechanical properties of the genetically engineered bone.22

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Address reprint requests to: Gregory A. Helm, M.D., Ph.D., De-

partment of Neurosurgery, Box 212, Health Sciences Center, Char-

lottesville, Virginia 22908. email: ga9r@virginia.edu.