Bone morphogenetic protein gene therapy for the induction of spinal arthrodesis

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Gene therapy has many potential applications in neurosurgery. One application involves bone morphogenetic protein-2 (BMP-2), a low-molecular-weight glycoprotein that induces bone formation in vivo. Numerous studies have demonstrated that the BMP-2 protein can enhance spinal fusion. This study was undertaken to determine whether direct injection of an adenoviral construct containing the BMP-2 gene can be used for spinal fusion. Twelve athymic nude rats were used in this study. Recombinant, replication-defective type-5 adenovirus with a universal promoter and BMP-2 gene (Ad-BMP-2) was used. A second adenovirus constructed with a universal promoter and ß-galactosidase (ß-gal) gene (Ad-ß-gal) was used as a control. Seven and one-half microliters of virus was injected percutaneously and paraspinally at the lumbosacral junction in three groups (four animals each): 1) Ad-BMP-2 bilaterally, 2) Ad-BMP-2 on the right, Ad-ß-gal on the left, and 3) Ad-ß-gal bilaterally. Computerized tomography (CT) scans of the lumbosacral spine were obtained at 3, 5, and 12 weeks. At 12 weeks, the animals were killed for histological inspection. Ectopic bone formation was seen both on three-dimensional CT reconstruction and histologically in all rats at sites treated with Ad-BMP-2. Histological analysis revealed bone at different stages of maturity adjacent to the spinous processes, laminae, and transverse processes. This study clearly demonstrated that it is possible to produce in vivo endochondral bone formation by using direct adenoviral construct injection into the paraspinal musculature, which suggests that gene therapy may be useful for spinal fusion in the future.

Key Words * gene therapy * spinal fusion * bone morphogenetic protein * percutaneous * adenovirus * animal model * rat

The use of osteoinductive proteins to promote or enhance spinal fusion is attracting great interest in the basic science and clinical communities.[4,5,7,9,15,18,19,21,22,24,25] Although internal fixation devices have been developed that can successfully achieve short-term stabilization at virtually all levels of the spine, long-term stability requires bone fusion, typically using autologous bone grafts as the osteoinductive material. Although autografts are currently the "gold standard" for initiating bone fusion in the clinical setting, the addition of osteoconductive matrices and osteoinductive growth factors to the autograft are currently being investigated. Bone morphogenetic proteins (BMPs) have been shown to be
extremely effective in enhancing bone deposition at fusion sites in a variety of animal models and are currently being tested in Phase I clinical trials for the treatment of spondylolisthesis. The BMPs comprise a family of proteins within the transforming growth factor-ß superfamily based on amino acid homology.[2,3,13,14] When applied in vivo, many of the BMPs induce ectopic bone formation by a characteristic pathway.[17] Pluripotent mesenchymal cells are initially stimulated to proliferate and differentiate into chondrocytes, the cartilaginous tissue then calcifies and is invaded by vascular channels, and this eventually progresses to mature bone containing normal marrow spaces. Although a single application of BMP may be adequate to enhance spinal arthrodesis, this approach is limited by BMP's short-term bioavailability and the current inability to control bone deposition over time. These two obstacles could be overcome by using gene therapy techniques to introduce the BMP gene into cells at the fusion site to achieve long-term, controllable BMP expression.

The most successful technique for the introduction of therapeutic genes into cells in vivo is cellular transduction using viral vectors.[1,8,10,11,12,20,23] Although retroviruses, adeno-associated viruses, lentiviruses, and herpes viruses are all actively being investigated, adenoviruses are advantageous because of their high transduction rates and high production titers. However, adenoviruses can induce a strong immune response that may limit the duration of gene expression in vivo, even in nondividing cells.

We have shown in a previous study that a replication-defective adenovirus containing the BMP-2 gene can induce bone formation when injected percutaneously into the thigh musculature of nude rats (Fig. 1). The new bone was formed by endochondral mechanisms and limited to the injection site, without evidence of systemic toxicity. Ossicles could be clearly seen at the treatment sites on three-dimensionally (3D) reconstructed computerized tomography (CT) scans 3 weeks after treatment, which increased in density during the following weeks. The present study was performed to determine whether percutaneous delivery of this BMP-2 adenoviral vector can be used to induce endochondral bone formation adjacent to the spine and to evaluate this technique for use in spinal fusions.
Fig. 1. Diagram illustrating the mechanism of endochondral bone formation after administration of Ad-BMP-2.

CLINICAL MATERIAL AND METHODS

Animal Selection
Because the host immune responses may limit transgene expression in adenoviral vectors, 12 athymic Sprague-Dawley nude rats were used for this study.[6] These rats do not respond to T cell-dependent mitogens present when adenoviruses infect normal cells.

Adenoviral Constructs
We used recombinant adenovirus type 5 with the BMP-2 gene under the control of a cytomegalovirus (CMV) promoter (Ad-BMP-2). In vitro analysis has demonstrated the virus' capability to produce BMP-2 in cells transfected in tissue culture (Pittman D, personal communication, 1997). Another recombinant type 5 adenovirus with the β-galactosidase (β-gal) gene under the control of the CMV promoter (Ad-β-gal) was used as a control. Both of these adenoviruses 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. These viruses were stored until use at -80°C in 10% phosphate buffered saline at a concentration of 5 X 10^8 particles/µl.

Injection Procedure
The 12 rats were anesthetized with chloral hydrate, and the lumbosacral area was prepared in a sterile fashion. The rats were divided into three groups of four animals each and underwent paraspinal, percutaneous injection at the lumbosacral junction with 7.5 µl of virus. The virus was injected as follows: 1) Ad-BMP-2 bilaterally, 2) Ad-BMP-2 on the right, Ad-β-gal on the left, and 3) Ad-β-gal bilaterally. To place the virus in the proper location, 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 viral solution (3.75 X 10^9 particles) was injected.

Imaging Analysis
The animals were sedated and CT scans of the lumbosacral junction were obtained at Weeks 3, 5, 8, and 12 postinjection. Axial 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 3D reconstruction was performed using a Voxel Q workstation.

Histological Analysis
At 12 weeks postinjection, the rats were anesthetized and underwent transcardial perfusion with 100 ml of phosphate buffered saline followed by 350 ml of a 0.05% glutaraldehyde solution. The lumbosacral spines were harvested and decalcified with 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 10-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 cover slipped.
Sources of Supplies and Equipment

The recombinant adenovirus type 5 with the **BMP-2** gene was generously provided by Genetics Institute, Andover, MA and that with **ß-gal** was a gift from Dr. C. Kao of the University of Virginia Cancer Center. The ethylenediaminetetraacetic acid was obtained from Stephens Scientific Company, Riverdale, NJ. Both the Picker PQ-2000 CT scanner (version 4.2) and the Voxel Q workstation were acquired from Picker International, Cleveland, OH.

RESULTS

**Imaging Analysis**

At 3, 5, 8, and 12 weeks postinjection, CT scans revealed ectopic bone formation adjacent to the spinous processes and lamina at each of the Ad-BMP-2 injection sites, but no radiographic changes occurred at the control Ad-ß-gal injection sites (Fig. 2). The three-dimensionally reconstructed CT scan is shown in Fig. 3. The spinal canal showed no evidence of ectopic calcification within and no growth of bone from outside in.

![CT scans of the spine at 12 weeks](image)

**Fig. 2.** Two-dimensional CT scans of the spine at 12 weeks. Left: Control rat with no evidence of bone formation or changes in spine or surrounding muscle and connective tissue. Note spinous process (S) and vertebral body (B). Right: Treated rat injected with Ad-BMP-2 on right. Note large fusion mass (F) adjacent to the spinous process (S) and lamina (L).
Fig. 3. Three-dimensionally reconstructed CT study of a treated rat spine at 12 weeks. Upper: Note the large fusion mass (F) nested between the spinous processes (S). Lower: The fusion mass (F) extends from the spinous process to involve the facets (J) laterally.

**Histological Analysis**

Histological examination of sections of the Ad-BMP-2 injection site 12 weeks postinjection revealed extensive endochondral bone formation within the paraspinal musculature (Fig. 4). There were large areas of mature bone containing developed vascular channels as well as areas of cartilage. The new bone was in solid continuity with the adjacent laminae, the inferior facet, and spinous process. Interestingly, the focal bone deposition had sharp borders, without evidence of extensive diffusion into the surrounding muscle or spinal cord. There was no evidence of an inflammatory response at the injection site or pathological changes in the surrounding muscle fibers.
DISCUSSION

In this study we have demonstrated that gene therapy techniques can be used to express BMP-2 in the paraspinal region, leading to endochondral bone formation. There was no evidence of bone formation distant from the injection site and no evidence of neural compromise, suggesting that this approach may be safe to use in the clinical setting. It is noteworthy that the bone formed in a discrete mass at the injection site and did not form along the needle tract. This may be because of the method of injection through a guide needle. It could also be due to the bioactivity of the BMP-2 protein. At femtomolar concentrations this agent promotes chemotaxis, and at nanomolar concentrations mesenchymal cell proliferation predominates. It is not until micromolar concentrations are used that BMP-2 promotes bone differentiation.[16] Therefore, bone formation is induced at the injection site but not along the needle tract. Another interesting observation is that at 3 months postinjection there continued to be cartilagenous tissue at the injection site, which may be interpreted in several ways: there could be persistent expression of BMP-2 leading to continued endochondral bone formation 3 months after injection, which may
eventually calcify to form mature bone; it is also possible that using this technique, BMP-2 expression will only initiate endochondral bone formation, leaving some new tissue in the cartilagenous state. The addition of other bone morphogenetic vectors may be required to promote calcification of the persistent cartilage.

This novel gene therapy technique may have numerous applications in spine stabilization procedures. The BMP-2 adenoviral vector could be added to autologous bone grafts to enhance bone deposition at the fusion site. Therefore, BMP gene therapy could have the potential to improve long-term stability significantly after spine instrumentation and limit the need for extensive harvesting of autograft from the iliac crest or fibula. Alternatively, percutaneous injection of the vector into the paraspinal musculature, facet joints, or the annulus fibrosis could fuse the spine in a minimally invasive fashion. Finally, percutaneous injection of the vector into spinal fractures could increase the rate of healing and decrease the risk of nonunion and spinal instability.

Although this study clearly demonstrates the potential use of this therapeutic approach in spinal fusions, many issues need to be addressed before this approach can be used clinically. Although promoting spinal arthrodesis by using gene therapy may be possible in small animals, it is unclear whether this technique will induce adequate bone growth in large animals and humans. Furthermore, because the bone-inductive properties of BMPs vary in different species, it is unclear which BMPs will be active in primates. It is possible that a cocktail of bone-inductive vectors may maximally stimulate bone formation. In addition, newer-generation viral vectors may be more suitable for use in humans. The required duration of BMP expression, the minimal cellular transduction rate, and the effects of the immune response against the vector all need to be studied in detail.

The use of tissue-specific promoters may make it possible to target transgene expression to specific cell types, in addition to limiting systemic gene expression. We are currently constructing two new adenoviral vectors containing the osteocalcin and actin promoters. The osteocalcin promoter should transcriptionally target BMP expression to osteoblasts, which may be useful in the treatment of osteopenic processes such as osteoporosis. The actin promoter should limit BMP production to muscle cells and may, therefore, be useful in promoting spinal fusions when injected into the paraspinal musculature. Inducible promoters could also be used to upregulate BMP expression until adequate bone formation has been achieved or to downregulate BMP production to prevent exuberant bone formation and potential neural compromise. Finally, detailed vector dosing and biomechanical testing studies need to be performed to define further the role of gene therapy in the treatment of spine disease.

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