Enhancement of Tendon-Bone Integration of Anterior Cruciate Ligament Grafts with Bone Morphogenetic Protein-2 Gene Transfer

A HISTOLOGICAL AND BIOMECHANICAL STUDY

BY VLADIMIR MARTINEK, MD, CHRISTIAN LATTERMAN, MD, ARVYDAS USAS, MD, STEVEN ABRAMOWITCH, BS, SAVIO L-Y. WOO, PHD, FREDDIE H. FU, MD, AND JOHNNY HUARD, PHD

Investigation performed at the Growth and Development Laboratory, Children's Hospital of Pittsburgh, and the Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania

Background: The integration of tendon grafts used for replacement of the anterior cruciate ligament is still sometimes unsatisfactory and may be associated with postoperative anterior-posterior laxity. The goal of this study was to examine the capacity of bone morphogenetic protein-2 (BMP-2) gene transfer to improve the integration of semitendinosus tendon grafts at the tendon-bone interface after reconstruction of the anterior cruciate ligament in rabbits.

Methods: The anterior cruciate ligaments of adult New Zealand White rabbits were replaced with autologous double-bundle semitendinosus tendon grafts. The semitendinosus tendon grafts had been infected in vitro with adenovirus-luciferase, adenovirus-LacZ (AdLacZ), or adenovirus-BMP-2 (AdBMP-2); untreated grafts served as controls. The grafts were examined histologically at two, four, six, and eight weeks after surgery. In additional experiments, the structural properties of the femur-anterior cruciate ligament graft-tibia complexes, from animals killed eight weeks postoperatively, were determined from uniaxial tests. The stiffness (N/mm) and ultimate load to failure (N) were determined from the resulting load-elongation curves.

Results: Genetically engineered semitendinosus tendon grafts expressed reporter genes as well as BMP-2 in vitro. The AdLacZ-infected grafts showed two different histological patterns of transduction. Intra-articularly, infected cells were mostly aligned along the surface, and they decreased in number between two and eight weeks after surgery. In the intra-tunnel portions of the grafts, the number of infected cells did not decrease during the observation period. Moreover, a high number of transduced cells was found in the deeper layers of the tendons. In the control group, granulation-type tissue at the tendon-bone interface showed progressive reorganization into a dense connective tissue, and a later establishment of fibers resembling Sharpey fibers.

In the specimens with an AdBMP-2-infected anterior cruciate ligament graft, a broad zone of newly formed matrix resembling chondro-osteoid had formed at the tendon-bone interface at four weeks after surgery. This area was increased at six weeks, showing a transition from bone to mineralized cartilage and nonmineralized fibro-cartilage. In addition, in the AdBMP-2-treated specimens, the tendon-bone interface in the osseous tunnel was similar to that of a normal anterior cruciate ligament insertion.

The stiffness (29.0 ± 7.1 N/mm compared with 16.7 ± 8.3 N/mm) and the ultimate load to failure (108.8 ± 50.8 N compared with 45.0 ± 18.0 N) were significantly enhanced in the specimens with an AdBMP-2-transduced graft when compared with the control values (p < 0.05).

Conclusion: This study demonstrates that BMP-2 gene transfer significantly improves the integration of semitendinosus tendon grafts in bone tunnels after reconstruction of the anterior cruciate ligament in rabbits.

Clinical Relevance: Novel technologies including gene therapy and tissue engineering, such as those described in this study, may provide useful therapeutic procedures to enhance biological healing after reconstruction of the anterior cruciate ligament.
raft integration is required for successful reconstruction of the anterior cruciate ligament. Solid graft incorporation after bone-to-bone healing within the osseous tunnels is one of the advantages of using bone-patellar tendon-bone grafts to replace the anterior cruciate ligament.

Supporters of this technique believe that the rigid osseous fixation and faster healing of bone-patellar tendon-bone grafts outweigh the potential disadvantage of greater donor site morbidity.

The integration of soft-tissue tendon grafts, such as quadruple semitendinosus-gracilis tendon grafts, in the bone tunnels is still critical to the success of both direct and indirect fixation methods. Direct implantation of tendon within a bone tunnel results in a fixation predominantly composed of fibrous tissue with collagen fiber bundles aligned along the load axis, which does not restore a normal anterior cruciate ligament insertion. In animal experiments, tendon grafts used to replace the anterior cruciate ligament were found to be surrounded by connective tissue up to twelve months after implantation, whereas a normal anterior cruciate ligament insertion can be re-established six months following reconstruction with a bone-patellar tendon-bone graft. In a meta-analysis of four studies with a minimum duration of follow-up of two years, patients treated with a bone-patellar tendon-bone graft had a greater chance of obtaining a stable knee and nearly a 20% greater chance of returning to their preinjury activity levels than did patients treated with a hamstring graft.

Recently published data indicate that the prevalence of bone tunnel enlargement is increased after reconstruction of the anterior cruciate ligament with a hamstring graft. However, the clinical effect of tunnel widening on knee stability is not yet clear, despite studies of the clinical results at two to three years postoperatively.

The goal of the present study was to develop a technique to improve the integration of semitendinosus tendon grafts in the tunnels after replacement of the anterior cruciate ligament. In the first part of the study, we evaluated the feasibility of in vitro gene transfer (with adenovirus-luciferase, adenovirus-LacZ, or adenovirus-bone morphogenetic protein-2 [AdBM-P-2]) to semitendinosus tendons used to replace the anterior cruciate ligament, and we attempted to demonstrate the expression of these marker genes in the grafts before and after implantation. In the second part, the gene transfer was used to transfer the BM-P-2 gene into tendon grafts for evaluation of its histological and biomechanical effects on the integration of the grafts in the tunnels after reconstruction of the anterior cruciate ligament in rabbits.

Materials and Methods

Forty-eight skeletally mature female New Zealand White rabbits were used in this study. The animals were kept in the animal facility of Children’s Hospital of Pittsburgh’s Rangos Research Center in accordance with the policies and procedures detailed in the Guide for the Care and Use of Laboratory Animals (United States Department of Health and Human Services). The research protocol was reviewed and approved by the Animal Research and Care Committee at the authors’ institutions.

Viral Vectors

The experiments were performed with replication-defective adenoviral vectors (first generation E1-E3 deleted) carrying the gene encoding bacterial β-galactosidase (LacZ) (GeneVec, Rockville, Maryland), the firefly luciferase gene, or the human BM-P-2 gene (Genetics Institute, Cambridge, Massachusetts) under the control of the human cytomegalovirus promoter.

In Vitro Marker Gene Expression

Both semitendinosus tendons from four New Zealand White rabbits were harvested and cut into four fragments. The tendon fragments (0.5 cm in length) were placed in tissue culture with Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in an atmosphere of 5% CO₂ and 95% air at 37°C. After twenty-four hours, ten tendon fragments were washed and were exposed to 3.0 x 10⁹ particles of adenovirus carrying the luciferase gene suspended in 100 µL of serumless medium (Neuman-Tytell; Gibco, Grand Island, New York), ten tendon fragments were exposed to 3.0 x 10⁹ particles of adenovirus carrying the bacterial β-galactosidase gene suspended in 100 µL of serumless medium, and ten tendon fragments were placed in 100 µL of serumless medium to serve as controls. Four hours after transduction, the conditioned medium was replaced and the tendon fragments were supplied with fresh medium every five days. Two tendon fragments in each group were harvested for histological analysis at twenty-four hours and at two, four, and six weeks.

The efficiency of viral transduction with adenovirus-luciferase was quantitatively assessed with use of a standard luciferase assay system. The tendon fragments were homogenized and were run through three freeze-thaw cycles. The luciferase luminescence (U/µg tissue) in the tissue of rabbit semitendinosus tendons after transduction with adenovirus-luciferase. The values after twenty-four hours and one, three, and six weeks in vitro are shown. *P < 0.001, compared with the control value. #P < 0.05, compared with the values at one, three, and six weeks.

Fig. 1
ciferase luminescence (units/mg tissue) of supernatant was measured in a luminometer with use of a luciferase assay according to the manufacturer's instructions (Promega, Madison, Wisconsin) and compared among the different groups with analysis of variance.

Expression of the LacZ gene leads to the synthesis of the enzyme β-galactosidase, and this can be detected by incubating samples with X-gal, which forms an intracellular blue product in the presence of β-galactosidase. The qualitative assessment of viral transduction with AdLacZ was performed on 7-μm cryostat sections of the tendons, which were incubated with X-gal for twenty-four hours at 37°C and counterstained with eosin.

In Vivo Marker Gene Expression

Graft preparation: The semitendinosus tendons were harvested forty-eight hours prior to the reconstruction of the anterior cruciate ligament; placed in tissue culture for twenty-four hours; and, according to group assignment, infected with viral particles or not infected.

Reconstruction of the anterior cruciate ligament: The animals were anesthetized, and the anterior cruciate ligament was exposed with a medial arthrotomy and was excised. Tibial and femoral tunnels (2.0 mm in diameter) were drilled at the anatomical insertions of the anterior cruciate ligament; the pretreated autologous double-bundle semitendinosus tendon graft was placed through the tunnels and tied with number-2-0 Ethibond sutures (Ethicon, Somerville, New Jersey) over metal buttons at both tunnel ends. The animals received perioperative analgesics (500 mg of Torbutrol [butorphanol tartrate] and antibiotics (100 mg of cefazolin) and were permitted unrestricted cage activity.

Histological study of AdLacZ and AdBMP-2 expression: The anterior cruciate ligaments were replaced with semitendinosus tendon grafts in twenty-four rabbits as described above. Twenty-four hours prior to surgery, ten semitendinosus tendons were transduced with $1.2 \times 10^{10}$ particles of AdBMP-2, and ten remained untreated to serve as controls. Eight weeks after surgery, both hindlimbs were harvested and were stored at −20°C until biomechanical testing. Additionally, five untreated knee joints and four in which the anterior cruciate ligament had been reconstructed immediately before the biomechanical testing were tested as described below.

After removal of all hardware and soft tissue, the femur...
anterior cruciate ligament graft-tibia complexes were fixed in specially designed clamps, allowing tensile loading along the long axis of the graft in a materials testing machine (model 4502; Instron, Canton, Massachusetts). A preload of 1 N was applied, and the reference position of the crosshead was set to 0 mm. Following cyclic preconditioning of the constructs between elongation limits of 0 and 0.75 mm (ten cycles at 5 mm/min), a load-to-failure test was performed at an elongation rate of 10 mm/min. Stiffness (N/mm) was calculated from the slope of the linear region of the load elongation curve between 1.5 and 2 mm of elongation. A Student t test was performed to determine the effects of healing on the structural properties of the treated and untreated femur-anterior cruciate ligament graft-tibia complexes.

In Vitro BMP-2 Secretion

In vitro BMP-2 secretion by the fibroblasts of rabbit semitendinosus tendons infected with AdBMP-2 was quantified. Biopsy specimens were taken from four control, nontransduced grafts and four AdBMP-2-transduced grafts and were placed in tissue culture until the cells had migrated from the tissue and proliferated. The cells from each population were plated in triplicate, incubated until reaching confluence, and counted, after which the medium was replaced with serumless medium. After sixteen, thirty-two, and sixty-four hours, the amount of BMP-2 in the medium was determined by ELISA (enzyme-linked immunosorbent assay), as described previously. Medium with known concentrations of BMP-2 was assayed to establish a standard curve. The concentration of BMP-2 was presented as nanograms of BMP-2 per 500,000 cells.

Results

In Vitro Studies

Luciferase assay: Luciferase activity was demonstrated in the transduced tendons during the six-week observation period (Fig. 1). The luminescence in the tendons decreased from a mean (and standard deviation) of 168 ± 16 U/µg at twenty-four hours to 30 ± 7 U/µg at six weeks following transduction. The control tendons showed no luciferase activity above the background level (0.03 ± 0.01 U/µg).

Histological evaluation of AdLacZ expression: β-galactosidase-positive cells were observed in the superficial cell layers of the transduced tendon fragments at all time-points tested. The expression of this gene declined between twenty-four hours (Fig. 2, A) and six weeks (Fig. 2, B). All control tendons were LacZ-negative.

BM-P-2 secretion: The BM-P-2 secretion by the transduced cells increased from a mean of 0.315 ± 0.06 ng of BM-P-2/500,000 cells at sixteen hours to 3.64 ± 0.43 ng of BM-P-2/500,000 cells at sixty-four hours. The measurement in the controls (background level) was 2.52 × 10⁴ ng of BM-P-2/500,000 cells (Fig. 3).

In Vivo Studies

Surgery-Related Considerations

All reconstructions of the anterior cruciate ligaments were performed without technical problems or postoperative infections.
Five reconstructions (two controls and three AdBMP-2-treated specimens) that failed or had a persistent effusion were excluded from the experiment. Two reconstructions (one control and one AdLacZ-infected specimen) with anterior-posterior laxity at the postmortem examination and one specimen with an elongated anterior cruciate ligament, but a normal gross appearance, were included in the histological examination.

Histological Evaluation of AdLacZ Expression
β-galactosidase-expressing cells with two different patterns of transduction were detected in the treated anterior cruciate ligament grafts. In the intra-articular portions of the tendon grafts, the number of transduced cells, which were mostly aligned along the surface of the tendons, decreased during the course of six weeks (Fig. 4, A and B). In the intra-tunnel portions, the number of LacZ-positive cells did not decline. Moreover, a large number of transduced cells were found in the deeper layers of the tendons at four weeks after surgery (Fig. 4, C). No LacZ-positive cells could be detected in the controls at any time-point (Fig. 4, D).

Histological Evaluation of AdBMP-2 Expression
Week two: In the controls, the interface between the implanted graft and the osseous tunnel was filled with poorly organized, highly cellular granulation-type tissue without continuity between the tendon and the adjacent bone (Fig. 5, A). The AdBMP-2-infected grafts showed a similar loose granulation-type tissue. However, a large number of activated osteoblasts was observed rimming the bone trabeculae adjacent to the implanted tendon (Fig. 6, A, arrows).

Week four: In the controls, the granulation tissue at the bone-tendon interface had developed into a dense connective tissue with poor vascularization. Some activated osteoblasts were seen rimming the adjacent bone trabeculae (Fig. 5, B, arrows). In the AdBMP-2-infected specimens, a broad layer of newly formed matrix resembling chondro-osteoid had been

Fig. 5
Photomicrographs of the tendon-bone interface of nontransduced grafts (controls) at two weeks (A), four weeks (B), six weeks (C and E), and eight weeks (D and F) following reconstruction of the anterior cruciate ligament. At two weeks, the interface between the implanted tendon graft and the bone was filled with a poorly organized, highly cellular granulation-type tissue (A) that progressively developed into a dense connective tissue eight weeks after surgery (D). Some osteoblasts (arrows) were seen rimming the adjacent bone trabeculae at four weeks (B) but were absent at six weeks after surgery (C). Some perpendicular collagen fibers at the interface between the tendon and the osseous tunnel could be observed at six weeks (C and E, arrows) and eight weeks (D and F, arrowheads) after surgery (hematoxylin and eosin; magnification, ×100 for A through D and ×200 for E and F).
Week six: In the control specimens, perpendicular collagen fibers were visible within the interface between the tendon and the osseous tunnel (Fig. 5, C and E, arrows). However, the activated osteoblasts that had been seen at four weeks were now absent (Fig. 5, C and E). In the AdBMP-2-infected specimens, there was a broad newly formed matrix showing a transition from fibrous to cartilaginous tissue with increasing mineralization toward newly formed bone, a process resembling endochondral ossification (Fig. 6, C).

Week eight: In the control specimens, there was further reorganization of the tendon-bone interface into a dense connective tissue. At this time-point, perpendicular collagen fibers were still noted between the graft and the osseous tunnel (Fig. 5, D and F, arrowheads). In the AdBMP-2-infected specimens, the zonal transition between the bone and tendon was even more pronounced than it had been at six weeks (Fig. 6, D).

No mononucleated cells infiltrated the graft, suggesting an absence of immunologic reaction or foreign-body response to the adenovirus. However, the immune response was not investigated in this study.

Biomechanical Testing
The stiffness (29.0 ± 7.1 N/mm compared with 16.7 ± 8.3 N/mm) and the ultimate load to failure (108.8 ± 50.8 N compared with 45.0 ± 18.0 N) were significantly enhanced in the femur-anterior cruciate ligament graft-tibia complexes with an AdBMP-2-transduced graft (p < 0.05) when compared with the values in the untreated controls (Table I). In all of the complexes with a transduced graft, the failure site was within the intra-articular portion of the graft, whereas in all controls, the failure mode was pull-out of the tendon from the tunnel. The normal femur-anterior cruciate ligament-tibia complexes demonstrated a significantly higher stiffness and ultimate load to failure than the experimental specimens (p < 0.05), whereas the specimens tested immediately after reconstruction displayed significantly lower values for these parameters than did the experimental specimens (p < 0.05) (Table I).

Discussion
In this study, a rabbit model was used to investigate the healing of anterior cruciate ligament grafts within the osseous tunnels. First, we developed an improved anatomical model of reconstruction of the anterior cruciate ligament, which consisted of a double-bundle semitendinosus tendon graft, anatomically placed, tightly fitted into the osseous tunnels, and fixed over buttons at each tunnel entrance. Single semitendinosus tendon grafts loosely pulled into osseous tunnels have failed intra-articularly at four or more weeks after reconstruction of the anterior cruciate ligament in rabbits8. The ex-
**TABLE I Biomechanical Properties of the Femur-Anterior Cruciate Ligament Graft-Tibia Complexes**

<table>
<thead>
<tr>
<th>Group</th>
<th>Stiffness* (N/mm)</th>
<th>Ultimate Load to Failure* (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 7)</td>
<td>16.7 ± 8.3</td>
<td>45.0 ± 18.0</td>
</tr>
<tr>
<td>AdBMP-2 (n = 6)</td>
<td>29.0 ± 7.1†</td>
<td>108.8 ± 50.8†</td>
</tr>
<tr>
<td>Immediately after reconstruction (n = 4)</td>
<td>4.0 ± 1.1</td>
<td>26.0 ± 13.0</td>
</tr>
<tr>
<td>Normal anterior cruciate ligament (n = 5)</td>
<td>45.3 ± 14.1</td>
<td>367.2 ± 49.0</td>
</tr>
</tbody>
</table>

*The values are given as the mean and standard deviation. †P < 0.05, compared with the control value.

tremely low ultimate load to failure of the femur-anterior cruciate ligament graft-tibia complexes in those experiments, which ranged from 2% to 6% of the values for normal complexes at four and five weeks after implantation, indicated that single-bundle tendon grafts were inadequate for replacement of the anterior cruciate ligament in rabbits. In comparison with the extra-articular models or the intra-articular one-bundle reconstructions of the anterior cruciate ligament24,25, the double-bundle grafts used in our study better simulate the situation in humans, in whom single-bundle tendon grafts are also insufficient for replacement of the anterior cruciate ligament. In our opinion, the increased diameter of the graft is not the only reason for its improved structural properties. Arnoczky et al. demonstrated that intimate contact and rigid fixation of tendon grafts also facilitated healing strength25. Kielty and Shuttleworth showed that physiological load and motion aid in repair, by causing fibroblasts and collagen fibrils to align parallel to the direction of force25. In view of the fact that all animals were fully weight-bearing within a matter of days and the semitendinosus tendon grafts were securely fixed in our experiment, a portion of the physiological load could be attributed to the reconstruction of the anterior cruciate ligament. In the future, this improved animal model might be considered for basic-science investigations of remodeling and integration of anterior cruciate ligament grafts.

Recently, BMP-2 has been used to enhance the integration of tendons in the bone tunnel. Rodeo et al. demonstrated an accelerated healing process of tendon to bone and a transitory increase in pull-out strength when BMP-2 had been applied to the tendon-bone interface in a dog model24. These findings correlated with the stimulation of osseous ingrowth found in their histological studies. Biomechanical testing revealed higher tendon pull-out strength in the BM-P-2-treated group at two weeks, but not at four, six, or eight weeks after surgery. Nicklin et al. found similar results when they used BMP-7 in an intra-articular tendon-bone healing model26. Exogenous application of BMP-7 upregulated the expression of BM-P-2 within fibroblasts and osteoblasts as well as bone formation at the tendon-bone interface of ovine anterior cruciate reconstructions. However, biomechanical testing at two, four, and six weeks showed no significant enhancement of the tendon-bone interface.

In the present study, transfer of the BMP-2 gene to grafts demonstrated biological and biomechanical effects of this osteoinductive protein on integration at the tendon-bone interface. We hypothesized that local expression of the BM-P-2 gene by the fibroblasts of the graft would provide a sustained and prolonged BMP-2 delivery system that was superior to the single application of the growth factor protein used in previous studies24,25. The accumulation of infected fibroblasts in the tunnels, combined with their ability to produce BM-P-2 after transduction with AdBMP-2, improved the integration of the anterior cruciate ligament grafts. This effect was demonstrated by both histological and biomechanical findings.

Histological analysis of the intra-tunnel portions of the implanted tendons showed an impressive effect of the transduction with AdBMP-2 on the characteristics of the tendon-bone interface of the implanted grafts. In the controls, the tendon-bone interface occurred by an intertwinement of the tendon to bone, as seen in our AdBMP-2-transduced specimens, was not seen in their experiment.

In the present study, transfer of the BMP-2 gene to the tendon-bone interface of the implanted grafts demonstrated biological and biomechanical effects of this osteoinductive protein on integration at the tendon-bone interface. We hypothesized that local expression of the BM-P-2 gene by the fibroblasts of the graft would provide a sustained and prolonged BMP-2 delivery system that was superior to the single application of the growth factor protein used in previous studies24,25. The accumulation of infected fibroblasts in the tunnels, combined with their ability to produce BM-P-2 after transduction with AdBMP-2, improved the integration of the anterior cruciate ligament grafts. This effect was demonstrated by both histological and biomechanical findings.

Histological analysis of the intra-tunnel portions of the implanted tendons showed an impressive effect of the transduction with AdBMP-2 on the characteristics of the tendon-bone interface of the implanted grafts. In the controls, the tendon-bone interface occurred by an intertwinement of the tendon to bone, as seen in our AdBMP-2-transduced specimens, was not seen in their experiment.

Collagenous fibers perpendicular to the load axis are considered biological anchors of anterior cruciate ligament
grants to the osseous tunnels in animals and humans\textsuperscript{9,25,27,28}. These collagenous structures have the appearance of the fibers described by Sharpey and Ellis—i.e., fibers perforating the periosteaum and extending from the tendon to the bone\textsuperscript{26}. With this indirect tendon-bone attachment, the collagenous fibers are perpendicular to the longitudinal load axis of the graft and are consequently stressed by bending forces when load is applied to the anterior cruciate ligament graft. This fixation is less stable than the direct anterior cruciate ligament insertion consisting of four transitional zones of tendon, non-mineralized fibrocartilage, mineralized cartilage, and bone, where parallel collagen fibers are anchored in the fibrocartilage and transmit longitudinal tensile forces well\textsuperscript{9,29,30}.

Follow-up reports and our own clinical experience have indicated that hamstring grafts exhibit some degree of increasing laxity more than two years after reconstruction of the anterior cruciate ligament\textsuperscript{11,31}. In addition, reconstruction of the anterior cruciate ligament with tendon grafts has been associated with tunnel widening\textsuperscript{12-15}. Although the etiology and the clinical relevance of this phenomenon are not yet clear, the possibility of failed tendon-bone integration and tunnel enlargement in such cases cannot be excluded, and in the future attention must be paid to the tendon-bone interface after reconstruction of the anterior cruciate ligament with hamstring tendons\textsuperscript{11,31}.

Before introducing gene transfer as a therapeutic method in orthopaedics, questions need to be answered regarding safety and regulatory issues\textsuperscript{32}. For this reason, this experiment should be considered as a pilot basic-science project that has demonstrated the feasibility of a gene transfer modality of treatment in orthopaedics.\textsuperscript{33}

\textbf{References}

22. Aronczyk SP, Toriglia PA, Warren RF, Allen AA. Biologic fixation of ligament