Biodegradable Gelatin Hydrogels Incorporating Fibroblast Growth Factor 2 Promote Healing of Horizontal Tears in Rabbit Meniscus

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Purpose: The purpose of this study was to investigate the in vivo effects of gelatin hydrogels (GHs) incorporating fibroblast growth factor 2 (FGF-2) on meniscus repair in a rabbit model. Methods: FGF-2 was biologically stabilized by incorporation into GHs. This system enables FGF-2 to be released with its biologic activity intact. A total of 64 skeletally mature female Japanese white rabbits were used. A horizontal tear was made in the medial meniscus, and these tears were divided into 4 groups: GH-FGF, GH-no FGF, FGF (FGF-2 alone), and no treatment. The meniscus was evaluated histologically at 2, 4, 8, and 12 weeks after surgery. Cell density and the percentages of proliferating cell nuclear antigen-positive cells and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells were measured, and a scoring system ranging from 5 points (complete healing) to 0 points (no evidence of healing) was used. Results: Cell density was significantly higher in the GH-FGF group than in the other 3 groups at 2, 4, 8, and 12 weeks (P < .01). The percentage of proliferating cell nuclear antigen-positive cells was significantly higher whereas the percentage of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells was significantly lower in the GH-FGF group at 2 and 4 weeks after surgery (P < .05). At 4, 8, and 12 weeks after surgery, healing scores were significantly higher in the GH-FGF group (2.5 points, 2.7 points, and 3.0 points, respectively) than in the GH-no FGF group (1.3 points, 1.4 points, and 2.0 points, respectively) (P < .05). Conclusions: GHs incorporating FGF-2 significantly stimulated proliferation and inhibited the death of meniscal cells until 4 weeks, thereby increasing meniscal cell density and enhancing meniscal repair in a rabbit model. Clinical Relevance: GHs incorporating FGF-2 are able to enhance the healing of meniscal injury.

Arthroscopic partial meniscectomy has been widely performed for the treatment of meniscal injury, despite the fact that meniscectomy alters the normal biomechanics of the knee and accelerates the development of osteoarthritis in the majority of patients. In an effort to preserve important structures in the knee, many surgeons are now considering primary repair of meniscal injury. However, the meniscus has a low healing potential, and in some cases primary repair has been unsuccessful. Several techniques have been developed to enhance the potential for meniscal healing, such as vascular access channels, synovial flap, fibrin clot, and hyaluronan, but these techniques are not commonly used. On the other hand, meniscal allografts have been performed clinically with an acceptable success rate, although there remains a potential risk of disease transmission from allograft tissue.

Several growth factors have been proven to be effective in enhancing meniscal tissue regenera-
tion. However, tissue regeneration by use of growth factors alone has not always been successful, because the half-lives of many growth factors are insufficient to sustain biologic activity. To overcome this problem, we developed a controlled-release system using gelatin hydrogel (GH). In this system growth factors are immobilized in GH through physicochemical interaction with gelatin molecules, and the immobilized growth factors are released from the hydrogel as a result of hydrogel degradation. Degradation can be controlled by changing the extent of cross-linking, which in turn produces hydrogels with different water contents.

Fibroblast growth factor 2 (FGF-2) is known to stimulate fibroblast proliferation and angiogenesis and to enhance collagen synthesis. Because the half-life of FGF-2 is very short (1.5 minutes), its application does not always result in successful tissue regeneration. Tabata et al. showed that FGF-2 was incorporated into the GH as a result primarily of electrostatic interaction, with the fluorescently labeled FGF-2 being homogeneously sorbed into the GH. When GHs incorporating FGF-2 were implanted, angiogenesis, adipogenesis, and bone regeneration were enhanced around implantation sites, in marked contrast to sites implanted with GHs free of FGF-2, as well as to sites injected with an aqueous solution of FGF-2. GHs incorporating FGF-2 have recently been applied clinically in patients with critical limb ischemia.

Okamura et al. reported a positive effect of FGF-2 on the reparatory process in canine meniscus. FGF-2 was injected into the peripheral synovium of the tear. In the FGF group, chondrocyte-like round cells appeared earlier than in the control group; however, there were no significant histologic differences when compared with the control group. Narita et al. were the first authors to address the effects of GHs incorporating FGF-2 on meniscus repair. They investigated the effects of GH-coated threads incorporating FGF-2 on human meniscal cells in an organ culture. The GH-coated threads incorporating FGF-2 stimulated the proliferation of meniscal cells and inhibited meniscal cell death, resulting in increased meniscal cell density. However, there have been no studies showing the in vivo effects of GHs incorporating FGF-2 on meniscus repair. Thus the purpose of this study was to investigate the in vivo effects of GHs incorporating FGF-2 on meniscus repair. In this study we examined healing of a horizontal tear in a rabbit model. We hypothesized that GHs incorporating FGF-2 would enhance cell proliferation and healing activity, as measured by cell density, and the percentage of proliferating cell nuclear antigen (PCNA)-positive cells and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL)-positive cells.

METHODS

Approval for the study was obtained from our institutional animal care and use committee.

Preparation of GHs Incorporating FGF-2

GHs were prepared by chemical cross-linking of aqueous gelatin solution with glutaraldehyde (GA), according to a previously reported method. Hydrogels used in this study were designed to release individual growth factors for a mean of approximately 1 week in the mouse back subcutis. Gelatin with an isoelectric point of 5.0 was prepared as an acidic gelatin. After we mixed 2.5 mmol of aqueous GA solution (Wako Pure Chemical Industries) with aqueous gelatin solution (5 wt %) preheated at 40°C, the mixed aqueous solution was cast into balance dishes and left for 12 hours at 4°C to allow for chemical cross-linking of gelatin. The resulting hydrogel sheets were placed in 100-mmol/L aqueous glycine solution and then agitated at 37°C for 1 hour to block the residual aldehyde groups of unreacted GA. Cross-linked hydrogel sheets were thoroughly washed with double-distilled water (DDW), freeze-dried, and sterilized with ethylene oxide gas. GH sheets were then cut into small squares (2 × 2 mm). Next, DDW containing 2 μg of human recombinant FGF-2 (2 μL) was added drop-wise onto freeze-dried GH sheets in the operating room under sterile conditions and then left for 1 hour to allow the hydrogel to be impregnated. The solution was completely sorbed into the hydrogel sheet within 1 hour, because the solution volume was much less than that theoretically required for the equilibrated swelling of hydrogels. Similarly, DDW (2 μL) was added drop-wise onto the GH sheet. Before the animal experiments, 2 types of GH sheets were prepared: GH sheets incorporating FGF-2 and GH sheets without FGF-2.

Animal Experiments

Sixty-four skeletally mature female Japanese white rabbits (Kumagai Shigeyasu, Miyagi, Japan) with a mean weight of 3.2 kg (range, 2.8 ± 3.3 kg) were used in this study. Each animal was anesthetized with an intramuscular injection of ketamine hydrochloride (35 mg/kg) and 2% xylazine hydrochloride (5 mg/kg).
Identical surgical techniques were used in both knees of each rabbit as follows. Rabbits were placed in the supine position. Through an aseptic technique, a medial parapatellar incision and arthrotomy were performed, the joint capsule was separated from the synovial tissues, and the medial collateral ligament was exposed. Synovial tissues were cut transversely and released from the medial meniscus. The peripheral rim of the meniscus was exposed. A horizontal tear (4 mm in width and 2 mm in length) was created in the medial meniscus with a 4-mm Beaver blade (BD Beaver; BD Biosciences, Franklin Lakes, NJ). The horizontal tear was placed 1 mm from the tibial surface and ran from the peripheral rim into the inner zone of the meniscus (Figs 1A, 1C). The horizontal tear was incomplete because the femoral and tibial surfaces of the meniscus were not injured.

The freeze-dried GH sheets were rehydrated through the drop-wise addition of DDW with and without FGF-2. A horizontal tear in the medial meniscus was filled with a prepared GH sheet incorporating FGF-2 solution (GH-FGF group), and the tear in the opposite side of the knee was filled with a GH sheet without FGF-2 (GH-no FGF group) (Fig 1B). Surgeons were blinded with regard to which GHs contained FGF-2.

Similarly, 2 groups were treated without GHs; the horizontal tear was injected with a 1-μg/L aqueous FGF-2 solution (2 μL) alone (FGF group) or with DDW (2 μL) alone (no treatment group). None of the injected fluid leaked from the injection sites. Surgeons were blinded with regard to which solution contained FGF-2.

Three rabbits were killed immediately after surgery, and 6 menisci were evaluated as time 0 specimens. Some samples were excluded from further evaluation because the horizontal tear penetrated the femoral or tibial surface. We therefore required more samples for detailed analyses.

After surgery, all rabbits were returned to their cages and allowed to move freely without joint immobilization. Rabbits were euthanized by intravenous injection of pentobarbital sodium immediately after surgery (3 rabbits) or at 2 weeks (15 rabbits), 4 weeks (17 rabbits), 8 weeks (15 rabbits), or 12 weeks (14 rabbits) postoperatively, and menisci were removed from the knee joint and processed for histologic evaluation.

**Histologic Evaluation**

Specimens were fixed in 10% formalin, embedded in paraffin, and cut into 4-μm slices along the radial plane. For histologic analysis, sections were stained with H&E. Histologic evaluation was performed in blinded fashion. Fourteen menisci were excluded from further evaluation because the horizontal tear penetrated the femoral or tibial surface. Further evaluation was performed for the remaining 114 menisci: 6 immediately after surgery, 26 at 2 postoperative weeks, 27 at 4 postoperative weeks, 28 at 8 postoperative weeks, and 27 at 12 postoperative weeks.
Horizontal tear

MM (radial plane)

1 3
2 4

FIGURE 2. Histologic specimens of medial meniscus showing areas selected for histologic evaluation (radial plane). All 4 areas (500 X 670 μm) adjacent to the tear were selected. The peripheral area (1, 2) was adjacent to the peripheral rim, whereas the inner area (3, 4) was adjacent to the edge of the tear.

To confirm the appropriateness of the horizontal tear, the length of the tear was measured by use of imaging software (Doctor File Viewer; Media Cybernetics, Bethesda, MD).

Cell density was examined immediately after surgery, as well as at 2, 4, 8, and 12 weeks postoperatively, in 2 peripheral and 2 inner areas adjacent to the horizontal tear, as shown in Fig 2. Each area was a square of 670 X 500 μm. Cell density data obtained in the 2 peripheral areas (areas 1 and 2 in Fig 2) were averaged, as were the cell density data obtained in the 2 inner areas (areas 3 and 4 in Fig 2). Cell density was examined in 1 section per specimen.

Reparative tissue was evaluated with a semiquantitative scoring system that was based on a previously reported scoring system.\(^2^2\)\(^2^3\) The total attainable score was 5 points, and scores were based on whether the horizontal tear was regenerated or filled with fibrous tissue.\(^2^2\) Five points were assigned to specimens when the tear had regenerated completely, with the appearance of normal meniscal tissue. Four points were assigned when the tear had almost fully regenerated but the location of the tear could be identified. Three points were assigned when the tear was entirely filled with fibrous tissue (Fig 3A). Two points were assigned when the tear was not entirely filled but fibrous tissue had infiltrated more than 25% of the tear (Fig 3B), and one point was assigned when fibrous tissue had infiltrated 25% or less of the tear (Fig 3C), because Arnoczky and Warren\(^3\) reported that vessels were found to originate in the perimeniscal capsular and synovial tissues and penetrate the peripheral 25% of meniscal tissues. Zero points were assigned to specimens when the tear showed no evidence of fibroblastic proliferation (Fig 3D). Semiquantitative scoring was performed in 5 sections taken 20 μm apart in the same specimen, and mean scores were used.

Immunohistochemistry for Detection of Proliferating Cells

To detect cell proliferation, PCNA staining was performed. After deparaffinization, binding activity of DNA-binding proteins was retrieved by microwave treatment.\(^2^4\) Sections were microwaved 3 times for 5 minutes each in 10-mmol/L citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3%
hydrogen peroxide in methanol for 10 minutes. The primary antibody was from mice. Anti-PCNA monoclonal antibody (PC10; Nichirei, Tokyo, Japan) was used as primary antibody, and sections were incubated with primary antibody at 4°C for 24 hours, followed by secondary antibody (biotinylated rabbit anti-mouse immunoglobulin G; Pharmingen [BD Biosciences]) for 60 minutes, and finally with an ABC kit (Vector Laboratories, Burlingame, CA). Immunoreactivity was visualized with a combination of 3,3-diaminobenzidine tetrahydrochloride and hydrogen peroxide. The percentage of PCNA-positive cells was examined immediately after surgery and at 2, 4, 8, and 12 weeks postoperatively in the peripheral and inner areas adjacent to the horizontal tear, as shown in Fig 2. PCNA-positive cells were examined in 1 section per specimen. We used goat serum as a control for antibody specificity.

**In Situ Detection of Cell Death**

For histochemical detection of meniscal cell death in the culture groups, TUNEL was carried out with an ApopTag peroxidase in situ apoptosis detection kit (Chemicon, Billerica, MA). After deparaffinization, sections were digested with proteinase K (20 µg/mL) for 15 minutes at room temperature. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 minutes. DNA was end labeled with digoxigenin-labeled dUTP by use of terminal transferase, and labeling was detected with a peroxidase-conjugated anti-digoxigenin antibody. Reactivity was visualized with a combination of diaminobenzidine and hydrogen peroxide. The percentage of TUNEL-positive cells was examined immediately after surgery and at 2, 4, 8, and 12 weeks postoperatively in the peripheral and inner areas adjacent to the horizontal tear, as shown in Fig 2. TUNEL-positive cells were examined in 1 section per specimen.

**Statistical Analyses**

After all of the previously mentioned examinations, the menisci treated with FGF-2 were revealed. Data on the length of the horizontal tear, cell density, and PCNA- and TUNEL-positive cells were then compared by analysis of variance, followed by Fisher post hoc testing. The Mann-Whitney test was used to compare histologic semiquantitative scoring between the GH-FGF group and GH-no FGF group, the GH-FGF group and FGF group, and the GH-FGF group and no treatment group. Differences of *P* < .05 were considered significant. Statistical analyses were performed with StatView for Windows, version 5.0 (SAS, Cary, NC).

**RESULTS**

**Evaluation of Horizontal Tear Length**

The length of the horizontal tear was 1.98 ± 0.14 mm in the GH-FGF group, 1.92 ± 0.16 mm in the GH-no FGF group, 2.01 ± 0.17 mm in the FGF group, and 1.95 ± 0.21 mm in the no treatment group. No significant differences were seen among the 4 groups (*P* > .05).

**Cell Density**

Immediately after surgery, cell density was 958.5/mm² in the peripheral area and 783.1/mm² in the inner area. Figure 4A shows the cell density in the GH-FGF, GH-no FGF, FGF, and no treatment groups. In the peripheral area, cell density was significantly higher in the GH-FGF group than in the other 3 groups at 2, 4, and 8 weeks after surgery (*P* < .01). In the inner area, cell density was significantly higher in the GH-FGF group than in the other 3 groups at 2 weeks after surgery (*P* < .01).

**Percentage of PCNA-Positive Cells**

Immediately after surgery, the percentage of PCNA-positive cells was 13.8% in the peripheral area and 8.4% in the inner area. Figure 4B shows the percentage of PCNA-positive cells in the GH-FGF, GH-no FGF, FGF, and no treatment groups. In the peripheral area, the percentage of PCNA-positive cells was significantly higher in the GH-FGF group than in the other 3 groups at 2 and 4 weeks after surgery (*P* < .01) (Figs 5A, 5B). In the inner area, no significant differences were seen among the 4 groups at any time point (*P* > .05).

**Percentage of TUNEL-Positive Cells**

Immediately after surgery, the percentage of TUNEL-positive cells was 28.8% in the peripheral area and 23.1% in the inner area. Figure 4C shows the percentage of TUNEL-positive cells in the GH-FGF, GH-no FGF, FGF, and no treatment groups. In the peripheral area, the percentage of TUNEL-positive cells was significantly lower in the GH-FGF group than in the other 3 groups at 2 and 4 weeks after surgery (*P* < .05) (Figs 5C, 5D). In the inner area, no significant differences were seen among the 4 groups (*P* > .05).
Histologic Semiquantitative Scoring

Figure 4D shows the semiquantitative scoring data for the 4 groups. Immediately after surgery, the histologic semiquantitative score was 0 in all samples. At 2 weeks after surgery, there were no significant differences in the 4 groups. At 4 and 8 weeks after surgery, however, the score was significantly higher in the GH-FGF group compared with the other 3 groups ($P < .05$). At 12 weeks after surgery, the score in the GH-FGF group was significantly higher than that in the GH-no FGF group ($P = .03$) (Fig 6). The mean score in the GH-FGF group tended to be higher than those in the FGF group and no treatment group, but the differences were not significant ($P = .07$ and $P = .05$, respectively).

DISCUSSION

We used PCNA to test for cell proliferation and TUNEL to test for cell death. In our study it was shown that GH incorporating FGF-2 significantly stimulated proliferation and inhibited the death of meniscal cells until 4 weeks, thereby increasing meniscal cell density and enhancing meniscal repair in a rabbit model.

Before this study, we conducted a pilot study in a 4-mm longitudinal tear in rabbit meniscus. We attempted to fill the tear with GH sheets and perform repair with a nylon thread. However, the rabbit meniscus was too small for this approach, and we were unable to secure the GH sheets within the longitudinal meniscal tear. Therefore we selected a horizontal tear.
model and found that the GH sheets remained in the horizontal tears, allowing the effects of GHs and FGF-2 to be ascertained.

FGF-2 stimulated both cell proliferation and glycosaminoglycan synthesis in a culture model of sheep meniscal cells. FGF-2 has also been identified as a survival factor (antiapoptotic factor). Our results suggest that GHs incorporating FGF-2 stimulate the proliferation of meniscal cells, thereby increasing their percentage. The significant diminution in TUNEL-positive cells was temporary.

Regionally specific healing of the meniscus is clinically well known, and these regional differences have mainly been attributed to blood supply, as suggested by the study of normal vascular anatomy of Arnoczky and Warren. Some biologic studies have also supported regional differences in meniscal cells with regard to mitotic potential and response to cyto-
kines. Arnoczky and Warren reported that vessels were found to originate in the perimeniscal capillary and synovial tissues and penetrate the peripheral 15% to 25% of meniscal tissues. Roeddecker et al. reported that the region at a distance of 1.5 mm from the capsular attachment was a poorly vascularized zone, where there is reduced healing potential. In our study the mean length of the horizontal tear was 1.97 mm, and the horizontal tears ran into the poorly vascularized zone of the meniscus, whereas the 2 tested inner areas were within the poorly vascularized zone.

Reparative changes were observed in the peripheral 25% of the meniscus in all 4 groups. On the other hand, in the GH-no FGF group, FGF group, and no treatment group, reparative changes in the inner zone were scarcely observed. In this study the percentages of PCNA-positive and TUNEL-positive cells showed significant differences in the peripheral areas at 2 and 4 weeks. Our data suggested that the FGF-2 incorporated into GHs primarily influenced meniscal cells in the peripheral zone, thereby enhancing meniscal repair not only in the peripheral zone but also in the inner zone. Therefore, in the FGF-GH group, reparative changes were observed in the inner zone.

In this study the amount of FGF-2 released from the GH sheets is unclear. The degradation period of hydrogels depends on their water content, which is a measure of the extent of hydrogel cross-linking: the higher the water content of hydrogels, the faster their in vivo degradation. Inoue et al. showed that FGF-2 administered to the knee joint cavity remained for 7 days in intra-articular injections of FGF-2 contained in GH microspheres. Kawai et al. showed that FGF-2-impregnated GH remained for 10 days in full-thickness skin defects in mouse backs. Tabata et al. reported that FGF-2 incorporated in acidic GHs maintains its biologic activity. In our blinded study, in the GH-FGF group, cell density at 2 and 4 weeks after surgery was significantly higher than that at 8 and 12 weeks after surgery. However, cell density in the GH-FGF group at 8 weeks was significantly higher than that in the GH-no FGF group, FGF group, and no treatment group. This suggests that FGF-2 incorporated into GH sheets also maintains its biologic activity and appears to have the potential to promote the repair of rabbit meniscus.

Cell density, the percentages of PCNA-positive cells and TUNEL-positive cells, and the histologic semiquantitative scores did not differ among the GH-no FGF, FGF, and no treatment groups in this study. This suggests that FGF-2 without GH provided no positive effects and the GH had no positive or negative effects, whereas the healing scores with FGF-2 incorporated into the GHs were significantly better when compared with the other groups.

This study had several limitations. First, 2 knees were used in each rabbit: 1 knee in the GH-FGF group and 1 knee in the GH-no FGF group in a set of rabbits and 1 knee in the FGF group and 1 knee in the no treatment group in the other set of rabbits. However, it is possible that the growth factors have a systemic effect. Second, we did not assess the extracellular matrix or biomechanical properties of the reparative tissue, nor did we obtain any quantitative data measuring FGF-2 release or concentrations. Third, we used horizontal tears that ran from the peripheral rim through about 50% of meniscal tissues, but clinically observed meniscal tears vary with regard to type, location, and size and the rabbit model itself may not correspond closely to human menisci.

GH sheets incorporating FGF-2 have been used clinically in other fields, and our study suggests that GH sheets incorporating FGF-2 are able to enhance the healing properties of the meniscus. However, further investigation is needed to evaluate long-term outcomes and biomechanical properties before GHs with FGF-2 can be clinically used for meniscal repair.

CONCLUSIONS

GHs incorporating FGF-2 significantly stimulated proliferation and inhibited the death of meniscal cells until 4 weeks, thereby increasing meniscal cell density and enhancing meniscal repair in a rabbit model.

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REFERENCES

Biomechanical Evaluation of an Anatomic Double-Bundle Posterior Cruciate Ligament Reconstruction

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Purpose: The purpose of this study was to evaluate the effect of the anatomic double-bundle reconstruction (ADBR) of the posterior cruciate ligament (PCL) with 2 femoral tunnels and 2 tibial tunnels. Methods: Eight fresh-frozen human knees were used. Bone tunnels were created based on the PCL anatomic footprints. A 9-mm looped semitendinosus and gracilis tendon for anterolateral bundle reconstruction (ALR), a 7-mm looped semitendinosus tendon for posteromedial bundle reconstruction (PMR), and the same grafts for the ADBR were used. Under a 100-N posterior tibial load and under a 100-N posterior tibial load and 5 Nm of external tibial torque, the posterior tibial translation (PTT) was measured. Results: Under posterior tibial load, at 0°, the PTT of the ALR was larger than that of the intact knee (P = .04) and the ADBR (P = .03); however, there were no significant differences between the PTT of the PMR and that of the ADBR (P = .28) and intact knee (P = .99). At 30°, the PTT of the ADBR was smaller than that of the ALR (P = .02) and PMR (P = .02). At 60°, the PTT of the PMR was larger than that of the ADBR (P = .02). At 90°, the PTT of the PMR was larger than that of the ADBR (P = .02). Under posterior tibial load and external tibial torque, at 0°, the PTT of the ALR was larger than that of the ADBR (P = .02). At 0° and 30° of knee flexion under the posterior tibial load and at 0° under the combination of posterior tibial load and external tibial torque, as well as better than the PMR at 30°, 60°, and 90° of knee flexion under the posterior tibial load. Clinical Relevance: The clinical outcome of PCL reconstruction might improve by reducing posterior knee laxity in knee extension with the ADBR.

Various anatomic and biomechanical studies indicated that the posterior cruciate ligament (PCL) is split into 2 functional bundles: the anterolateral (AL) bundle and the posteromedial (PM) bundle. These bundles are not a precisely anatomic separation, but they can be separated by their distinct patterns of tension at different knee flexion angles. The AL bundle is considered to form up to 85% of the cross section of the midsubstance of the PCL; however, the size of the femoral and tibial insertion area of the AL bundle is nearly equal to that of the PM bundle. The PM bundle is taut in knee extension and deep flexion, and the AL bundle is slack in knee extension, becoming tight in knee flexion. The conventional single-bundle PCL reconstruction attached importance to the AL bundle's function, because the AL bundle has a larger cross-sectional area and a significantly greater linear stiffness and ultimate load than the PM bundle. Because reconstruction of the AL bundle alone seems to be insufficient to reproduce the function of the native PCL, some investigators reported the clinical results of a double-bundle PCL reconstruction; however, the clinical results of PCL reconstruction have remained unpredictable.