The Effect of Growth Factors on Biomechanical Properties of the Bone–Patellar Tendon–Bone Graft After Anterior Cruciate Ligament Reconstruction: A Canine Model Study

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The Effect of Growth Factors on Biomechanical Properties of the Bone–Patellar Tendon–Bone Graft After Anterior Cruciate Ligament Reconstruction

A Canine Model Study

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Background: No studies have dealt with the effect of growth factors on the free tendon autograft in anterior cruciate ligament reconstruction.

Hypothesis: Application of exogenous transforming growth factor–β and epidermal growth factor may affect the structural properties and histology of the bone–patellar tendon–bone autograft after anterior cruciate ligament reconstruction.

Study Design: Controlled laboratory study.

Methods: Twenty dogs underwent anterior cruciate ligament reconstruction with the autogenous bone–patellar tendon–bone graft in bilateral knees. In 10 animals, 12 ng transforming growth factor–β and 300 ng epidermal growth factor mixed with fibrin sealant of 0.6 mL were applied to the left knee. In the remaining 10 dogs, fibrin sealant alone was applied to the left knee. No additional treatments were applied to the right knee.

Results: The growth factor application increased the stiffness and maximum failure load of the femur-graft-tibia complex at 12 weeks ($P = .016$ and $P = .012$, respectively); the sham treatment did not significantly affect them. Histologically, most of the cells in the grafts treated with growth factors had spindle-shaped nuclei; cells in the other grafts had round-shaped nuclei.

Conclusions: Application of transforming growth factor–β and epidermal growth factor improves the structural properties of the autograft after anterior cruciate ligament reconstruction in the canine model.

Clinical Relevance: Application of growth factors is a possible strategy to prevent graft deterioration in anterior cruciate ligament reconstruction.

Keywords: anterior cruciate ligament (ACL) reconstruction; bone-tendon-bone autograft; transforming growth factor–β (TGF-β); biomechanics; growth factor

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It has been established that structural properties of the femur-tendon autograft-tibia complex are reduced after ACL reconstruction and that the reduced properties are not restored even at 12 months after surgery.[7,10] The deterioration of the autograft is likely induced by extrinsic fibroblast proliferation and ingrowth along with revascularization, which occur after intrinsic fibroblast necrosis.[19,33] Therefore, one future goal in ACL reconstruction is

to prevent the graft deterioration after transplantation, or to accelerate mechanical restoration of the deteriorated graft. Recently, a number of in vitro studies have shown that application of various growth factors significantly affects fibroblasts harvested from tendon and ligament tissues. For example, transforming growth factor–ß (TGF-ß) increases both collagen and noncollagenous protein synthesis by fibroblasts[11,23,25,27] and stimulates fibroblast proliferation.[20,30,31] Epidermal growth factor (EGF) also stimulates fibroblast proliferation in vitro.[11,30] Combined application of these 2 growth factors enhances their effects.[4,28,32] In addition, several in vivo studies have reported that some growth factors significantly enhance healing of the injured medial collateral ligament.[7,14,21,37,40] Therefore, there is the possibility that intra-articular administration of these growth factors may affect remodeling of the graft in ACL reconstruction.

Recently, the authors[5,29] have studied the effect of TGF-ß and EGF on the in situ frozen-thawed rabbit ACL, an idealized model of anatomic but acellular autograft. We have found that intra-articular administration of TGF-ß and EGF not only significantly inhibits reduction of the material properties of the frozen-thawed ACL but also increases the cross-sectional area, and the administration changes the diameter distribution of collagen fibrils in the frozen-thawed ACL. However, this model is not completely equivalent to an ACL reconstruction model with the free tendon graft. No studies have dealt with the effect of growth factors on the free tendon autograft in ACL reconstruction.

Based on the above-mentioned studies,[5,29] we have hypothesized that an intra-articular application of TGF-ß and EGF mixed with the fibrin sealant may significantly affect the structural properties and histology of the bone–patellar tendon–bone (B-PT-B) autograft in ACL reconstruction. The purpose of this study is to test this hypothesis with a canine model.

MATERIALS AND METHODS

Study Design

A biomechanical study was conducted with 25 healthy, adult beagle dogs weighing 10.9 ± 0.6 kg (mean ± SD). Animal experimentation was carried out under the Role and Regulation of the Animal Care and Use Committee, Hokkaido University, School of Medicine. Twenty dogs underwent ACL reconstruction with the autogenous B-PT-B graft in bilateral knees (Figure 1). Then, in 10 of the 20 animals, 12 ng TGF-ß and 300 ng EGF (R&D System, Minneapolis, Minn) mixed with fibrin sealant of 0.6 mL (Kaketsuken Co, Kumamoto, Japan) were applied around the graft in the left knee (GF group), and fibrin sealant alone was applied as the sham treatment in the right knee (sham group). In the remaining 10 dogs, no additional treatments were applied after surgery, and only the right knee was used to obtain control data of natural ACL reconstruction with the B-PT-B autograft (control group). No animals were immobilized postoperatively, and all were allowed unrestricted activities in their cages (1 dog per cage). In addition, bilateral knees harvested from the remaining 5 dogs, which underwent no surgery, were used to obtain normal biomechanical data of the ACL. All animals were sacrificed by a lethal injection of thiamylal sodium at 12 weeks after the operation. In each group (n = 10), 8 and 2 knees were used for biomechanical and histological examinations, respectively.

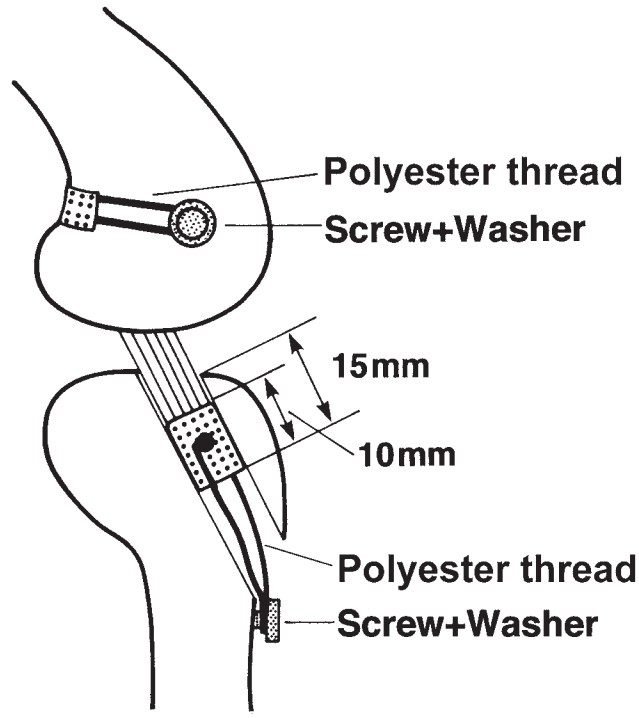

Figure 1. The ACL reconstruction procedures with the bone–patellar tendon–bone graft.

Growth Factors and a Delivery Vehicle

Recombinant human TGF-ß1 and recombinant human EGF were purchased from R&D Systems. Commercially available fibrin sealant (Kaketsuken Co) was used as a vehicle for the growth factors.[14,40] The fibrin sealant was formed by human fibrinogen (80 mg/mL) and thrombin being mixed in the presence of calcium and factor XIII. The TGF-ß1 and EGF were reconstituted according to the instructions given by the manufacturer. The reconstituted growth factors were mixed with the thrombin solution, and then the fibrinogen-thrombin solution was combined. The resultant fibrin sealant of 0.6 mL containing growth factors was applied to each knee.

Surgical Procedure and Postoperative Treatment

Each procedure was performed under general endotracheal anesthesia. Using sterile techniques, a medial parapatellar arthrotomy was performed in each knee. The anterior aspect of the infrapatellar fat pad was excised to allow

for a full visualization of the tibial attachment of the ACL. The ACL in both knees was excised.

The B-PT-B preparation having a width of 4 mm and a bone plug length of 10 mm was harvested from the right extensor apparatus of the knee, and the bone plugs were then trimmed so that the plug could be passed through a 4-mm-diameter sheath. The periosteum, which covered the anterior (ventral) aspect of the bone plug, was not dissected. A drill hole having a diameter of 1 mm was made in each bone plug, and a No. 1 nonabsorbable polyester suture (Ticron, Davis and Geck, Wayne, NJ) was passed through each hole. After the preparation, the cross-sectional area of the middle portion of each graft was measured with an area micrometer, which had been established in our previous studies.[22,26] In brief, the middle portion was located in the micrometer slot, and the plunger was placed on the specimen in the slot. The thickness of the specimen was measured while it was being pressed under a constant pressure of 0.12 MPa. The cross-sectional area of the middle portion was calculated by multiplying the slot width by the measured thickness.

The anteromedial surface of the tibia was exposed, and the periosteum was elevated. A bone tunnel having a diameter of 4 mm was drilled in the tibia through the central tibial insertion of the resected ACL to the exposed anteromedial surface of the tibia. Then, a small incision was made in the posterolateral part of the femoral joint capsule. A sharp curette was inserted from the incision to over the top of the lateral femoral condyle, and the cortical bone at this portion was then curetted along the over-the-top route so that a "trough" was created. For each graft, the distal end having a length of 15 mm was placed in the tibial bone tunnel, and the proximal end was placed in the trough. This over-the-top trough technique for the femoral side was performed because it had been used as a reliable and standard technique for the canine knee,[3,34] and the additional trough technique enabled this technique to resemble the tunnel technique for human patients. The tibial bone plug was grafted so that the periosteum surface faced the anterior wall of the tunnel. Consequently, the opposite surface of the bone plug faced the posterior wall. We tethered each end of the graft with the suture to a screw inserted into the bone, manually applying minimal graft tension in a direction aligned with the long axis of the bone tunnel with the knee flexed at 45° (Figure 1). After graft fixation, we found no anteroposterior instability by manual tests. The surgical wound was irrigated with a physiologic saline solution and closed with 3-0 nylon sutures.

None of the beagles were immobilized postoperatively. (70 cm in width, 68 cm in height, and 70 cm in depth). In this cage, each beagle could walk along the cage wall, although it could not run. At the time of sacrifice, first, anesthesia was induced by the intramuscular administration of ketamine hydrochloride (10 mg/kg). Then, a lethal dose of thiamylal sodium was injected intravenously.

Sample Preparation for Mechanical Testing

Each hindlimb for mechanical testing was wrapped in gauze moistened with physiologic saline solution and then wrapped in air-tight plastic films. The specimens were stored at –32°C until testing. All mechanical tests were performed blinded; namely, the researcher who performed the tests was different from the surgeon for this experimental operation, and the researcher was blinded concerning the group name of each specimen at the time of testing. Before mechanical testing, each knee was thawed overnight at 4°C. The knee was removed from the hindlimb, and the surrounding muscles were carefully removed.

Biomechanical Testing

The knee specimen with the femur having a length of 45 mm and the tibia having a length of 60 mm was removed from the animal immediately after sacrifice. Each specimen for biomechanical testing was wrapped in gauze moistened with physiologic saline solution and then wrapped in an air-tight polychiorovinylidene film. The specimens were stored at –32°C until the time of testing. Prior to mechanical testing, each knee was thawed overnight at 4°C. The anterior-posterior translation was evaluated according to Beynnon et al.[6] The specimen was mounted on the fixture of 5 degrees of freedom at 45° and 90° of knee flexion, and 50-N anterior and posterior shear loads were applied to the knee specimen.[17,18]

All soft tissues other than the graft were carefully dissected. Then the cross-sectional area of the graft was measured with the above-described area micrometer. The femur and the tibia were separately cast in rectangular aluminum tubes using polymethylmethacrylate resin.

With the use of a set of specially designed grips, the prepared femur-graft-tibia complex specimen was attached to a conventional tensile tester (PMT 250W; Orientec, Tokyo, Japan) so that the tibia was positioned to allow for tensile loading aligned with the long axis of the bone tunnel with the knee flexed at 45° (Figure 2). Two parallel lines were drawn transversely on the proximal bone surface of the femur and the tibia using Nigrosine stain as gauge-length markers to measure elongation of the femur-graft-tibia complex. Prior to the tensile test of the femur-graft-tibia complex, the specimen was preconditioned with a static preload of 0.5 N for 5 minutes, followed by 10 cycles of loading and unloading with a strain of 0.5% at a crosshead speed of 20 mm per minute.[17,18] Then, the femur-graft-tibia complex specimen was stretched until failure at the same rate, and the load-deformation curve was drawn on a recorder (X-Y-T Recorder 3023; Yokogawa, Tokyo, Japan). The elongation of the complex was determined with a video dimension analyzer (HTV-C1170; Hamamatsu Photonics, Tokyo, Japan) using the previously described gauge-length markers.[26] The specimen was kept moistened throughout the test period with a physiological saline solution spray. During tensile testing, failure modes were carefully observed in each femur-graft-tibia complex specimen. From the load-elongation curve, the linear stiffness was determined as the slope of the linear portion. In addition, the maximum failure load and the elongation at failure of the femur-graft-tibia complex were obtained on the load-elongation curve.

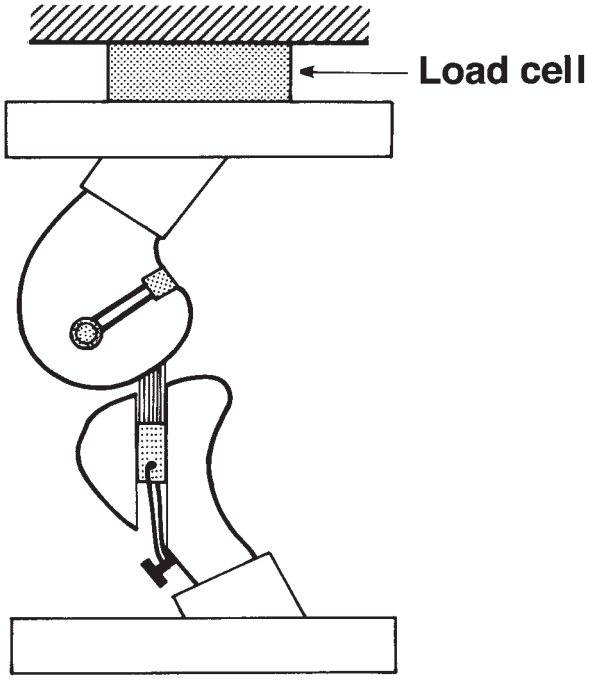

Figure 2. Tensile testing for the femur-graft-tibia complex.

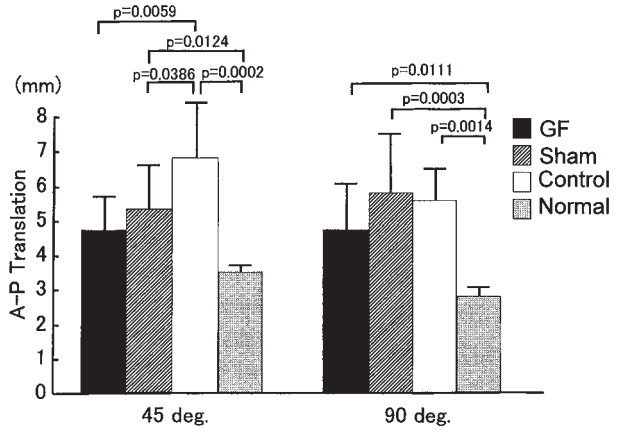

Figure 3. The anterior-posterior (A-P) translation of the tibia on the femur measured at 45° and 90° of flexion. There were no significant differences between the graft (GF) group and the normal knee, although the sham and control groups were significantly greater than the normal knees. There were no significant differences between the GF and sham groups.

TABLE 1
The Anterior-Posterior Translation of the Tibia at 12 Weeks (Mean ± SD)

	Group			
Knee Flexion Angle	Graft	Sham	Control	Normal
45°	4.7 ± 1.1	5.3 ± 1.4	6.8 ± 1.7	3.5 ± 0.4
90°	4.7 ± 1.3	5.8 ± 1.7	5.6 ± 1.1	2.8 ± 0.4

Histological Observation

The graft-tibia specimen for histological observation was fixed in a 10% buffered formalin solution immediately after harvesting from each limb. After the specimen was decalcified, it was cast in paraffin blocks. Each specimen was sectioned parallel to the longitudinal axis of both the tendon graft and the bone plug, stained with hematoxylin and eosin and toluidine blue for light and polarized light microscopy.

Statistical Analysis

All data were described with the mean and SD values. Each biomechanical parameter was compared with the 1-way analysis of variance. The chi-square test was used to compare the failure mode between the groups. The most essential comparison was made between the GF and sham groups to test the above-described hypothesis. To support this comparison, additional comparisons were performed between the sham and control groups and between the GF group and the normal knee. The significance level was set at $P = .05$ for each test. The statistical software (Stat View, Abacus Ontent Inc, Berkeley, Calif) was used for the analysis.

RESULTS

Gross Observations

Postoperatively, the animals carried the involved limbs for about a week. Their cage activities gradually increased, and by the fourth postoperative week, the animals showed only a mild degree of lameness. No lameness was noted after the sixth week. At the time of sacrifice, no graft failure was found in any of the knees. The intra-articular graft was enveloped by granulation tissues, which adhered to the infrapatellar fat pad, in each group. The adhesion appeared to an obviously greater degree in the GF and sham groups than in the control group. The synovial membrane was also thickened around the patellofemoral joint to an obviously greater degree in the GF and sham groups than in the control group. Neither meniscus tears nor tibiofemoral osteophyte formation was found in each knee. In addition, a small part of the surface of articular cartilage showed mild degeneration, such as softening and fibrillation, in each knee.

Anterior-Posterior Translation of the Tibia

Concerning the anterior-posterior translation of the tibia on the femur measured at 45° of flexion, there were no significant differences between the GF and sham groups (Figure 3). However, the sham group (average 5.3 mm) as well as the control group (6.8 mm) were significantly greater ($P = .012$ and $P = .001$, respectively) than the nor-

TABLE 2
The Cross-sectional Area and the Structural Properties of the Femur-Graft-Tibia Complex Measured at 12 Weeks (Mean ± SD)

	Group			
Femur-Graft-Tibia Complex	Graft	Sham	Control	Normal
Cross-sectional area (mm^2)	12.7 ± 2.1	9.6 ± 2.0	10.8 ± 3.6	9.8 ± 1.6
Stiffness (N/mm)	93.8 ± 20.1	62.1 ± 32.1	54.4 ± 17.8	130.4 ± 35.6
Maximum load (N)	303.2 ± 107.5	184.4 ± 90.4	176.4 ± 74.1	626.0 ± 108.5
Elongation at failure (mm)	4.7 ± 1.0	4.7 ± 0.8	4.9 ± 1.4	7.0 ± 2.1

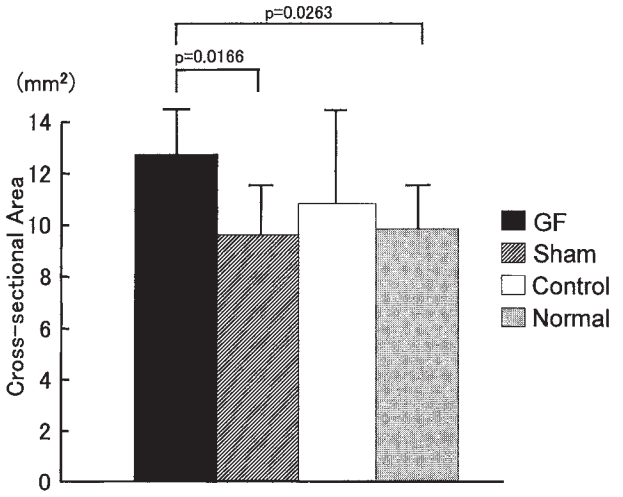

Figure 4. The cross-sectional area of the tendon graft measured at 12 weeks. The area was significantly greater in the graft (GF) group than in the sham group, whereas there were no significant differences between the sham and control groups. Note that the area measured immediately before implantation averaged 4.9 ± 0.5 mm^2.

TABLE 3
Failure Modes in Tensile Tests

	Group			
Mode	Graft	Sham	Control	Normal
Midsubstance tear	4	8	8	0
Avulsion fracture	4	0	0	10

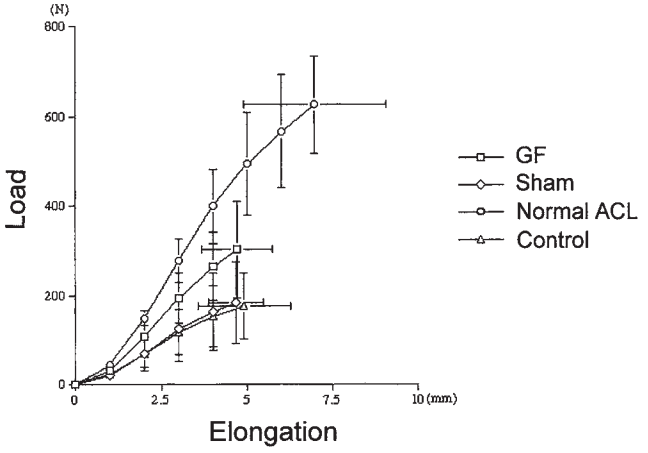

Figure 5. The load-elongation curves of the femur-graft-tibia complex in graft (GF), sham, and control groups and the normal knee.

mal knees (3.5 mm), whereas there were no significant differences between the GF group (4.7 mm) and the normal knee. Concerning the translation measured at 90° of flexion, we found the same tendency as observed at 45°, except that the GF group was also significantly greater than the normal knees (Table 1).

Cross-sectional Area of the Graft

The cross-sectional area of the tendon graft measured immediately before implantation averaged 4.9 mm^2 with a SD of 0.5, which was approximately half that of the normal ACLs (average 9.9 mm^2, SD ± 1.2). In each group, the cross-sectional area significantly increased twice or more at 12 weeks, compared to the preoperative value. The area was significantly greater (P = .017) in the GF group (12.7 mm^2) than in the sham group (9.7 mm^2) at 12 weeks, whereas there were no significant differences between the sham and control groups (Figure 4). In addition, the cross-sectional area of the GF group was significantly greater (P = .026) than that of the normal ACLs (Table 2).

Structural Properties of the Femur-Graft-Tibia Complex

Concerning failure modes of the femur-graft-tibia complex, the tendon graft portion was torn at the midsubstance in 4 knees and avulsed from the tibia with a small fragment in the remaining 4 knees in the GF group. The remaining part of the bone plug that had been grafted within the tunnel was not identified from the tibia. In the sham group, the tendon graft portion was torn at the midsubstance in each knee. There were significant differences in the failure

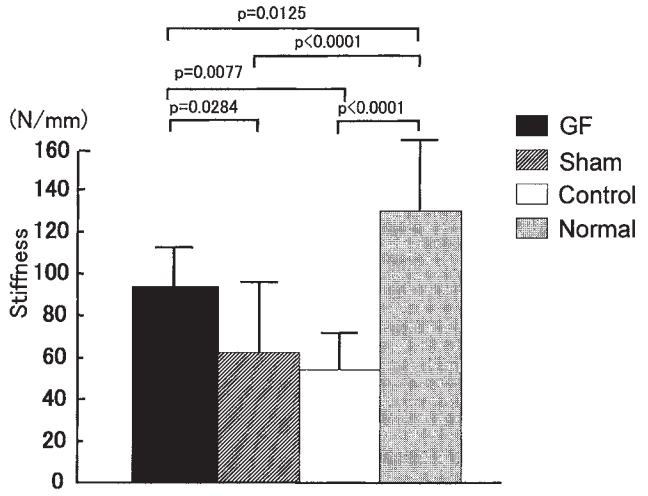

Figure 6. The stiffness of the femur-graft-tibia complex measured at 12 weeks. The graft (GF) group was significantly greater than the sham group, whereas it was significantly lower than the normal knees. There were no significant differences between the sham and control groups.

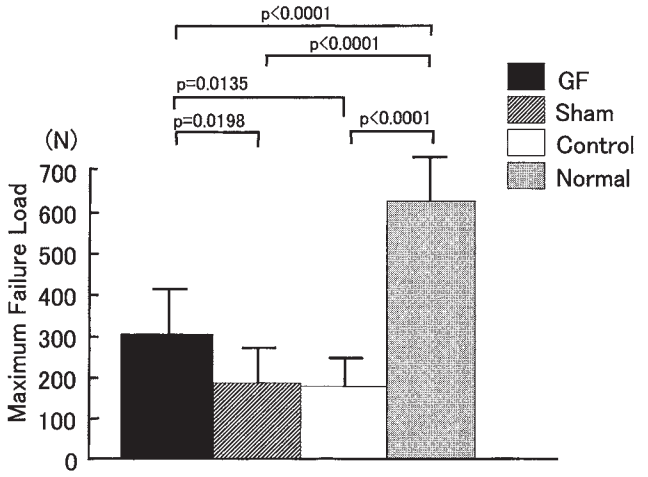

Figure 7. The maximum failure load of the femur-graft-tibia complex measured at 12 weeks. The graft (GF) group was significantly greater than the sham group, whereas it was significantly lower than the normal knees. There were no significant differences between the sham and control groups.

mode between the 2 groups (chi-square test, $P = .025$). In the control group, the graft portion was torn at the midsubstance in each knee, whereas the normal ACL was avulsed from the tibia with a small fragment in each knee (Table 3). The size of the avulsed fragment was similar between the GF group and the control group.

Figure 5 shows the load-elongation curves of the femur-graft-tibia complex in the GF, sham, and control groups and the normal knee. The load-elongation curve of the GF group was obviously different from not only the curves of the sham and control groups but also the curve of the normal knee.

The stiffness of the femur-graft-tibia complex in the GF group (average, 93.8 N/mm) was significantly greater ($P = .016$) than that in the sham group (62.1 N/mm) at 12 weeks, whereas it was significantly lower ($P = .013$) than that of the normal knees (130.4 N/mm). There were no significant differences between the sham and control groups (Figure 6).

Concerning the maximum failure load of the femur-graft-tibia complex, the GF group (average, 303.2 N) was significantly greater ($P = .012$) than the sham group (184.4 N) at 12 weeks, whereas it was significantly lower ($P < .0001$) than that of the normal knees (626.0 N). There were no significant differences between the sham and control groups (Figure 7).

Concerning the elongation at failure of the femur-graft-tibia complex, there were no significant differences between the GF, sham, and control groups. Each group was significantly lower than the normal knees ($P = .003$, $P = .003$, and $P = .007$, respectively) (Table 2).

Histologic Observation of the Graft

The collagen fibers in the GF group appeared to be more oriented compared to those in the control and sham groups (Figures 8 A, B, and C). The latter fibers appeared to be coarse (Figures 8 B and C). In the control and sham groups, numerous cells that had a round-shaped nucleus were randomly scattered in the midsubstance of the graft (Figure 8 B and C). In the GF group (Figure 8A), almost all of the cells had a spindle-shaped or oval-shaped nucleus. In this group, the number of cells scattering in the midsubstance appeared to be less than that in the control and sham groups. In the GF group, in addition, we observed several cells with an oval-shaped nucleus that were longitudinally aligned between collagen fascicles, parallel to the fibers (Figure 8A). However, the histology involving cells and collagen fibers of the GF group was different from that of the normal ACL (Figure 8D).

Observations using transillumination by polarized light showed that a regular crimp pattern of collagen fibers with relatively large amplitude and wavelength was predominantly observed in the GF group (Figure 9A). In the control and sham groups, a crimp pattern was irregular (Figures 9 B and C). However, the crimp pattern of the GF group was different from that of the normal ACL, which showed regular crimp with amplitude and wavelength less than those of the GF group (Figure D).

DISCUSSION

This study clearly demonstrated that intra-articular administration of 12 ng TGF-ß and 300 ng EGF mixed with fibrin sealant significantly increases the cross-sectional area of the tendon graft, significantly changes the failure mode of the femur-graft-tibia complex, and significantly increases the stiffness of the femur-graft-tibia complex in the canine ACL reconstruction model. Histologically, this

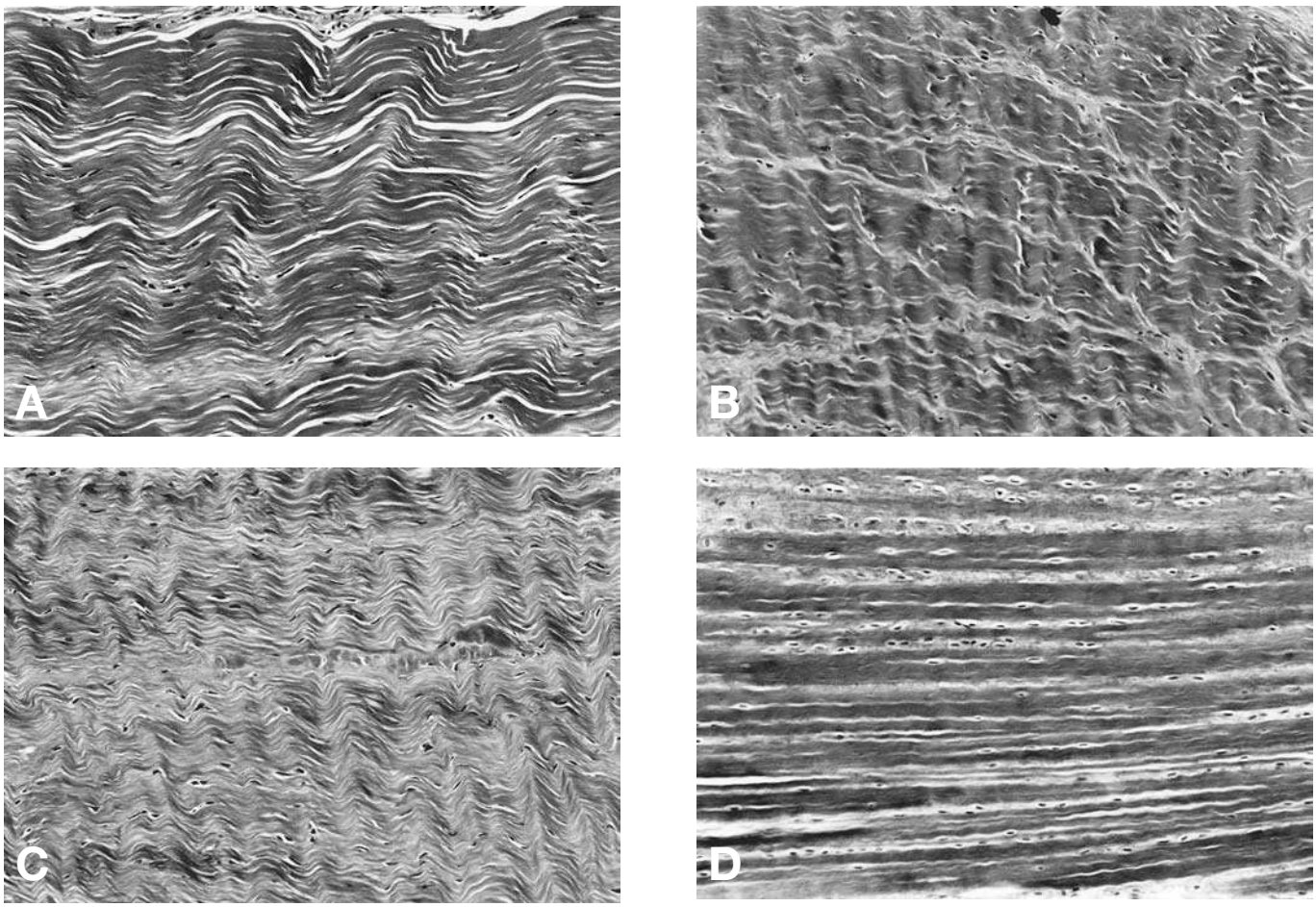

Figure 8. Histology of the midsubstance. The collagen fibers in the graft (GF) group (A) appeared to be more oriented compared to those in the control group (B) and the sham group (C). The latter fibers appeared to be coarse. Note the number, shape, and alignment of cells of the GF group compared to the control and the sham groups (see text). However, the histology of the GF group was different from that of the normal ACL (D).

study implied that the administration of these growth factors affects cell maturation in the tendon graft. Thus, this is the first article that reported that the intra-articular administration of TGF-ß and EGF significantly affects the structural properties of the femur-autograft-tibia complex reconstructed in ACL reconstruction with the B-PT-B graft.

A number of studies have shown the natural course of the tendon autograft after ACL reconstruction.[1-3,9,10,19] Following transplantation, intrinsic fibroblasts in the tendon graft undergo a process of ischemic necrosis. The graft is then enveloped with a synovial tissue by 2 weeks, and extrinsic cell infiltration with revascularization occurs in the graft. Butler et al[7] and Clancy et al[10] reported that the cross-sectional area significantly increases at approximately 6 weeks after ACL reconstruction surgery and that the structural properties of the femur-graft-tibia complex dramatically decrease throughout the observation period, compared to the normal opposite knee. Ultrastructurally, the population of small-diameter fibrils increases in the frozen-thawed goat ACL at 6 months.[13,16] In the present study, the histological and biomechanical results observed in the control group at 12 weeks were similar to the natural course of the patellar tendon graft reported in the above-described studies. Therefore, we can say that the control data in this study were not unusual, compared to the above-described studies with a canine model.

Concerning the effect of fibrin sealant, previous studies have reported that the application of fibrin sealant did not give any significant effects on remodeling of the frozen-thawed ACL[5,29] or the injured ligament.[14,40] The present study also showed that application of only fibrin sealant had no significant effects on structural properties of the femur-graft-tibia complex and histology of the graft. This fact also showed that the data in the sham group were not unusual in comparison with the above-described studies. However, the adhesion between the graft and the infrapatellar fat pad appeared to an obviously greater degree in the GF and sham groups than in the control group. The synovial membrane was also thickened around the patellofemoral joint to an obviously greater degree in the GF and sham groups than in the control group. These find-

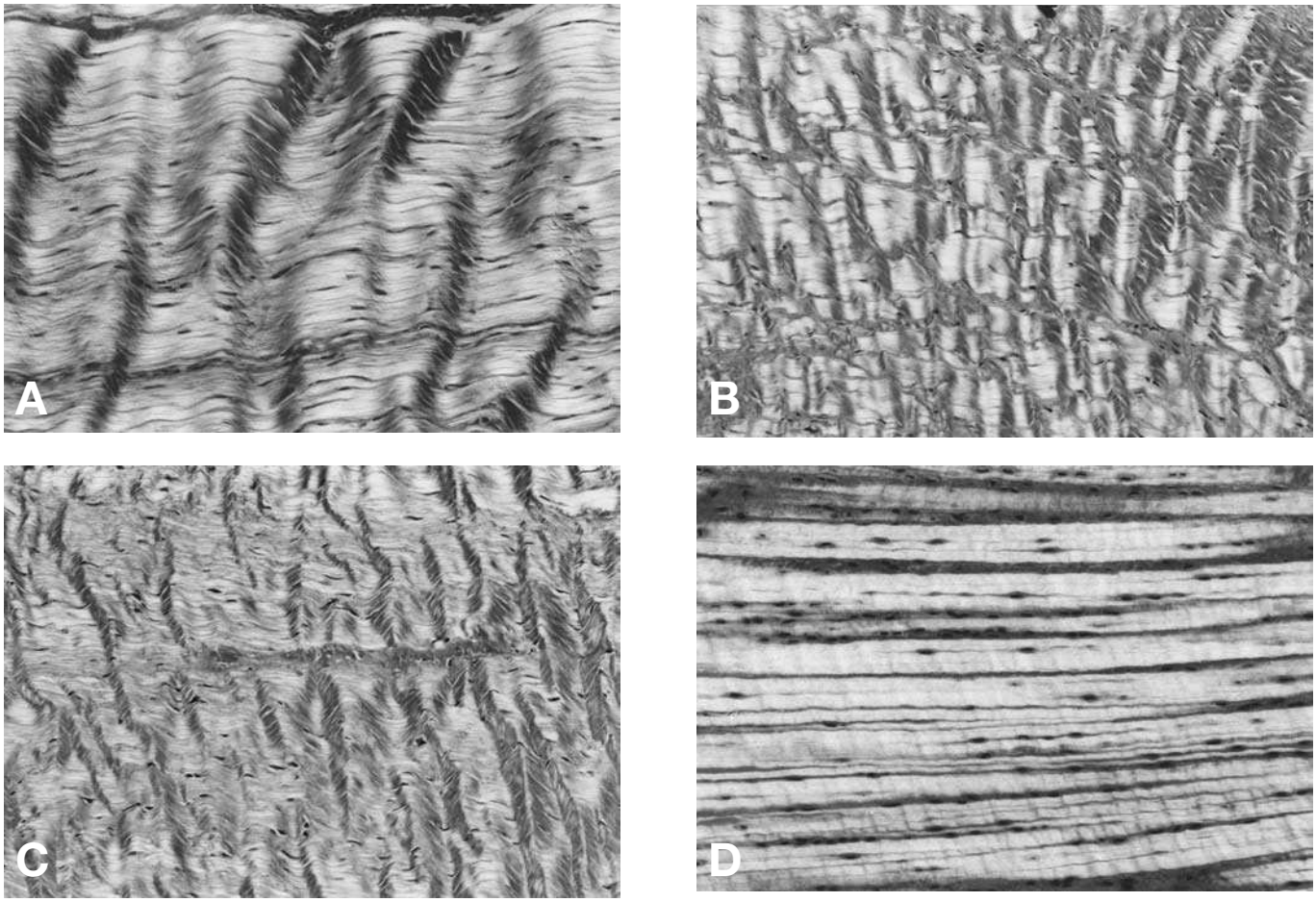

Figure 9. Observations using transillumination by polarized light. In the graft (GF) group (A), a regular crimp pattern of collagen fibers with relatively large amplitude and wavelength was predominantly observed. In the control group (B) and the sham group (C), a crimp pattern was irregular. However, the crimp pattern of the GF group was different from that of the normal ACL (D).

ings imply that the fibrin sealant, which was formed by human fibrinogen and thrombin being mixed, gave some adverse effects to the fat and synovial tissues, although it did not significantly affect the biomechanical data. In addition, we should recognize the possibility that TGF-ß and EGF may have detrimental effects on the tissues within the joint. For example, Van Beuningen et al[36] and Hulth et al[15] reported that repetitive application of TGF-ß into the knee joint induces degenerative changes in the cartilage. In this study, however, we applied these growth factors only once after surgery. This may be the reason we did not find any significant detrimental effects in this study.

Based on these fundamental results, the present study showed that the intra-articular application of TGF-ß and EGF with fibrin sealant significantly affects the biomechanical properties of the femur-graft-tibia complex after ACL reconstruction. For example, the graft was avulsed from the tibia with a small fragment in 4 of the 8 knees in the GF group during tensile tests, whereas each complex failed at the midsubstance of the graft in the sham and control groups. The avulsion from the tibia with a small fragment is a common failure mode in the normal ACL.[38,39] In this study, the size of the avulsed fragment was similar between the GF group and the control group, and the remaining part of the bone plug that had been grafted within the tunnel was not identified from the tibia. In this study, therefore, we estimated the bone plug to be healed within the tunnel. Our previous study with the same canine model showed that the bone plug was healed within the tibial tunnel at 6 weeks after surgery.[34] This study supports our estimation on bone plug healing. In addition, the maximum failure load of the femur-graft-tibia complex of the GF group was significantly greater than that of the sham group. It was noted that the growth factor application significantly increased the stiffness of the femur-graft-tibia complex, compared to the sham and control groups. This result was considered to show the stiffness of the graft substance to a greater degree than that of the insertion of the graft. Thus, these results suggested that the application of TGF-ß and EGF significantly suppresses the natural deterioration of the structural properties of the tendon autograft in ACL reconstruction.

We should speculate on a few possible mechanisms to explain why the structural properties of the graft were

changed by the application of TGF-ß and EGF, although we did not definitely determine them in this study. First, in the present study, the administration of TGF-ß and EGF significantly increased the cross-sectional area of the tendon graft. Marui et al[23] reported that TGF-ß increases not only collagen synthesis but also noncollagenous protein synthesis in ligament fibroblasts. Therefore, there is the possibility that in the present study, an application of the growth factors enhanced synthesis of new tissues involving various collagens, bonding proteins, and/or proteoglycans in the graft. These changes might affect the proteoglycan-collagen network and/or minor collagen network in the graft. An increase of the cross-sectional area does not always mean an increase of the structural properties. In this study, however, for example, if causes of the increase of the cross-sectional area involved enhanced production of type III and/or type I collagens in comparison with the natural course, it is considered that it was one of the causes of the increase of the structural properties. Second, histological observations in this study implied that the administration of these growth factors affects cell maturation in the tendon graft. We should note in the natural course of the graft that the structural properties of the graft are extremely deteriorated after surgery. A number of previous studies have reported that TGF-ß suppresses collagenase activities from fibroblasts in various tissues.[24,35] In the present study, therefore, there is the possibility that an application of the growth factors suppressed the collagenase activity from extrinsic fibroblasts infiltrating in the graft, resulting in the inhibition of their mechanical deterioration. These mechanisms can simultaneously occur. Further studies should be conducted to clarify the mechanisms or causes of the increase of the structural properties of the graft.

Concerning the anterior-posterior translation of the knee, all the operated knees appeared to be looser than the normal knee. It has been well known that this phenomenon commonly occurs in animal models.[7,10,16] It shows that the graft is elongated to some degree in the animal model. Therefore, we should recognize that the natural course of the graft is not completely the same as that in human patients. However, we also note that results from such animal models have contributed to the progression of the ACL reconstruction. In the present study, although the sham and control groups were significantly greater than the normal knees, there were no significant differences between the GF group and the normal knee. Therefore, although there were no significant differences between the GF and sham groups, some tendencies exist that indicate the application of TGF-ß and EGF improved the translation. However, we could not come to a conclusion about the effect of the application of TGF-ß and EGF on the length of the graft.

It is necessary to recognize some limitations concerning the experimental model used in this study. First, we used the beagle model in this study. The average maximum load of the normal ACL was 626 N. This value is higher than the value of approximately 400 N shown in previous studies with beagles.[12] Therefore, we believe that our testing condition was acceptable. However, this value was less than the maximum load of 1210 N reported with German shepherd dogs.[8] Among various canine models, this model is recommended for use in experimental studies from an ethical viewpoint. However, we should recognize that the beagle model is relatively small in various canine models. Second, we used the bilateral injury model. In this model, it is considered that animals have to evenly load the graft in each knee during walking. This is an advantage of this model but simultaneously is a disadvantage. Namely, there is a possibility that the graft might be overloaded in the early phase after ACL reconstruction and that the overstress might give detrimental effects to the graft, such as an increase of the anterior-posterior translation of the knee.[17] However, the bilateral injury model enabled us to compare the 2 groups under the same postoperative environment concerning the effect of the growth factor application, which was the main purpose of this study.

There are some additional limitations to this study. The first limitation was that we could not determine the structural and mechanical properties of the graft itself, although we dealt with the whole femur-graft-tibia complex. However, the structural properties of the whole femur-graft-tibia complex are the most essential factors to evaluate the knee with ACL reconstruction from the clinical viewpoint, because not only the graft midsubstance but also insertion of the graft are equally important for success of ACL reconstruction. The second limitation was that we did not evaluate long-term results after ACL reconstruction. However, we believe that this study is of value because it first demonstrated that the application of TGF-ß and EGF significantly affects the biomechanical properties of the free tendon graft in ACL reconstruction. The third limitation was that we did not examine the diameter of collagen fibrils with a translucent electron microscope. The fourth limitation is a potential bias in the histological observations in this study. The sample number was only 2 in each group. In addition, our observation was traditionally performed according to the previous studies.[1,3,9,10,16] Therefore, we did not perform random sampling or observation in a blinded fashion, nor did we carry out statistical analyses on histological observations. Based on the biomechanical data with the statistical significance, we should deal with the histological findings as additional data supporting the biomechanical data. Further studies should be conducted with a sufficient number of more appropriate animals to answer questions concerning each limitation.

Finally, it has been recognized that there are various biological and biomechanical factors affecting the outcome of ACL reconstruction.[41] This study also suggested that growth factors are important factors affecting results of ACL reconstruction. As to the clinical relevance of this study, this study implied that the application of certain growth factors is of value to be studied as one of the strategies for future ACL reconstruction to prevent graft deterioration after transplantation or to accelerate mechanical restoration of the deteriorated graft. However, we also should recognize the possibility that growth factors may

have some detrimental effects on the tissues within the joint, such as detrimental effects of TGF-ß on the joint cartilage. Therefore, in the near future, we should study how to effectively apply such growth factors to the targeted tissue without their detrimental effects.

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