

# Effects of Administration of Exogenous Growth Factors on Biomechanical Properties of the Elongation-type Anterior Cruciate Ligament Injury With Partial Laceration

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**Background:** No studies have been conducted to clarify the in vivo effect of growth factor application on healing in the injured anterior cruciate ligament.

**Hypothesis:** Administration of exogenous growth factors significantly increases the structural properties of the injured anterior cruciate ligament.

**Study Design:** Controlled laboratory study.

**Methods:** Thirty-six rabbits were randomly divided into 4 groups of 9 animals each after an overstretched injury was made in the right anterior cruciate ligament. In group 1, no treatment was applied around the injured anterior cruciate ligament. In group 2, 0.2 mL fibrin sealant was applied around it. In group 3, 4 ng transforming growth factor- $\beta$ 1 mixed with 0.2 mL fibrin sealant was applied. In group 4, 20  $\mu$ g platelet-derived growth factor-BB mixed with 0.2 mL fibrin sealant was applied. Each rabbit was sacrificed at 12 weeks after the surgery. In addition, 9 knees randomly harvested from all the left knees were used to obtain normal control data. The femur-anterior cruciate ligament-tibia complex specimens were biomechanically and histologically evaluated.

**Results:** Concerning the maximum load and the stiffness, group 3 was significantly greater than groups 1 and 2, whereas there were no significant differences among groups 1, 2, and 4. Groups 1, 2, 3, and 4 were significantly lower than the control group.

**Conclusions:** The application of 4 ng transforming growth factor- $\beta$ 1 significantly enhances healing in the injured anterior cruciate ligament.

**Clinical Relevance:** Administration of certain growth factors is of value to be studied as one of the future therapeutic options for the overstretched anterior cruciate ligament injury.

**Keywords:** anterior cruciate ligament (ACL); biomechanics; elongation; growth factor; ligament healing

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Overstretched ACL injury with partial midsubstance laceration frequently occurs in various types of athletic accidents.<sup>10</sup> For this type of ACL injury, we have had no therapeutic options for repairing the ligament injury itself because of the poor healing potential of the ACL. Previous in vitro studies, however, showed that some growth factors, such as transforming growth factor (TGF), platelet-derived growth factor (PDGF), and so on, can stimulate

fibroblast proliferation and enhance collagen and noncollagenous protein synthesis by fibroblasts.<sup>6,13,16,23,24</sup> Authors of recent *in vivo* studies have reported that such growth factors enhance healing in the injured medial collateral ligament (MCL).<sup>3,9,14,34,35</sup> In addition, authors have shown that intra-articular application of TGF- $\beta$ 1 significantly affects the extrinsic fibroblasts that infiltrate the ACL graft.<sup>2,22,26,37</sup> Therefore, there is the strong possibility that a therapeutic application of some specific growth factors can enhance healing in the overstretched ACL injury. No studies, however, have been conducted to clarify the *in vivo* effect of growth factor application on healing in the overstretched ACL injury because there have been no appropriate models of such an ACL injury. Recently, however, we have established a new overstretched ACL injury model with partial midsubstance laceration and permanent elongation, which can be created with a quantitative technique.<sup>12</sup>

The purpose of this experimental study was to evaluate the *in vivo* effect of intra-articular administration of TGF- $\beta$ 1 and PDGF-BB on the knee laxity and structural properties of the elongation-type ACL injury with partial laceration.

## MATERIALS AND METHODS

### Study Design

A total of 36 skeletally mature female Japanese White rabbits weighing 3.5 (SD = 0.2) kg were used in this study. Animal experiments were carried out at the Institute of Animal Experimentation, Hokkaido University School of Medicine, under the Rules and Regulations of the Animal Care and Use Committee. In each animal, the right ACL was injured using the below-described quantitative technique under general anesthesia and aseptic conditions. Then, the animals were randomly divided into 4 groups of 9 animals each. In group 1, no treatment was applied around the injured ACL. In group 2, 0.2 mL fibrin sealant (Kaketsuken Co, Kumamoto, Japan) was applied around the injured ACL as the sham treatment. The fibrin sealant was chosen as a delivery vehicle according to previous studies.<sup>2,9,22</sup> In group 3, 4 ng recombinant human TGF- $\beta$ 1 (R&D Systems, Minneapolis, Minn) mixed with 0.2 mL fibrin sealant was applied around the injured ACL. In group 4, 20  $\mu$ g recombinant human PDGF-BB (R&D Systems) mixed with 0.2 mL fibrin sealant was applied around the injured ACL. This was the first study that intended to clarify the effects of TGF- $\beta$ 1 and PDGF-BB on the *in vivo* ACL injury model, so no previous data were available to us to decide on an effective dose of the growth factors for the ACL injury. Therefore, the dose of growth factors (4 ng TGF- $\beta$ 1 and 20  $\mu$ g PDGF-BB) in the present study was chosen because it was the most effective *in vivo* for healing of the MCL injury according to the study reported by Hildebrand et al.<sup>9</sup> In addition, the dose of TGF- $\beta$ 1 was effective *in vivo* for remodeling of the ACL graft,<sup>2,22</sup> and the dose of PDGF-BB was effective *in vitro* for fibroblasts

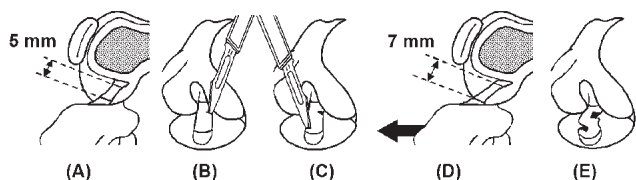
derived from the ACL.<sup>16,23,24</sup> All animals were sacrificed with an overdose of intravenous barbiturate at 12 weeks after surgery. In addition, 9 knees randomly harvested from all the left knees were used to obtain normal control data (control group). In each group, 7 of the 9 rabbits were used for biomechanical evaluation, and the remaining 2 were used for histological observation.

### Surgical Procedure and Intraoperative and Postoperative Treatments

Surgery was performed under anesthesia induced by an intravenous injection of pentobarbital (25 mg/kg). In each animal, the right knee was positioned at 90° of knee flexion by stabilizing the lower leg attached to an operating table. In a sterile fashion, the ACL was exposed through the medial parapatellar approach. Two marker lines were drawn on the surface of the ACL using a black stain (nigrosine). One of the 2 lines was 2 mm distal to the femoral attachment of the ACL, and the other line was 2 mm proximal from the tibial attachment. The normal length of the ligament was approximately 10 mm.

The right ACL was injured using the following quantitative technique.<sup>12</sup> An anterior drawer force of 10 N was applied to the tibia at 90° of knee flexion (Figure 1A), then the distance between the 2 lines was measured with a vernier caliper (Mitutoyo, Kanagawa, Japan). The distance was approximately 5 mm. The anteromedial and posterolateral halves for the ACL were transected with a scalpel at the proximal and the distal one-third levels, respectively, between the 2 marker lines (Figures 1 B and C). Then, a surgeon manually increased the anterior drawer force, monitoring the intermarker length, which was continuously measured by an assistant surgeon. When the length was elongated by 2 mm from the initial length, the surgeon maintained the elongation length for 5 minutes (Figure 1D). The ACL became slack after the anterior drawer force was removed (Figure 1E). Finally, the length between the 2 marker lines was measured again under the 10-N anterior drawer force. The measured length was increased by 1.0 mm (SD = 0.1), 19.8% (SD = 2.0) from the initial length.

TGF- $\beta$ 1 and PDGF-BB were reconstituted according to the instructions given by the manufacturer. Pure fibrin sealant, which was commercially available for human treatment, was purchased from Kaketsuken Co. The preparations consisted of fibrinogen (80 mg/mL) dissolved in an aprotinin solution (1 mL) at 37°C. A second mixture of powdered thrombin (250 IU) dissolved in 1 mL of calcium chloride solution (5.9 mg/mL) was prepared separately. The mixture was drawn into separate 1-mL syringes and placed in a dish that allowed simultaneous mixing. Thus, the fibrin sealant did not contain any other proteins, including cytokines or growth factors. In groups 2, 3, and 4, 0.2 mL fibrin sealant with or without the growth factor was applied around the injured ACL. The fibrin sealant was scooped with a small round spoon and was put into the intercondylar space. Subsequently, the whole intercondylar space was filled with the sealant. The sealant did



**Figure 1.** Operative treatment to create the elongation-type ACL injury with laceration. A, the distance between the 2 lines was measured at 90° of knee flexion under an anterior drawer force of 10 N. The distance was approximately 5 mm. B and C, the anteromedial and posterolateral half of the right ACL was transected with a scalpel at the proximal and distal one-third levels, respectively. D, a surgeon manually applied an anterior drawer force, monitoring the length between the 2 marker lines, so that the ACL was elongated 2 mm for 5 minutes. E, the ACL became slack after the anterior drawer force was removed.

not flow out from the space during surgery because it was a gel. Thus, the sealant was uniformly applied around the ACL.<sup>2,22</sup>

The incised joint capsule and the skin wound were closed in layers with 3-0 nylon sutures, and an antiseptic spray dressing was applied. No immobilization was applied after surgery, and animals were allowed unrestricted daily activities in their cages (52 cm in width, 35 cm in height, and 33 cm in depth).

### Tensile Testing

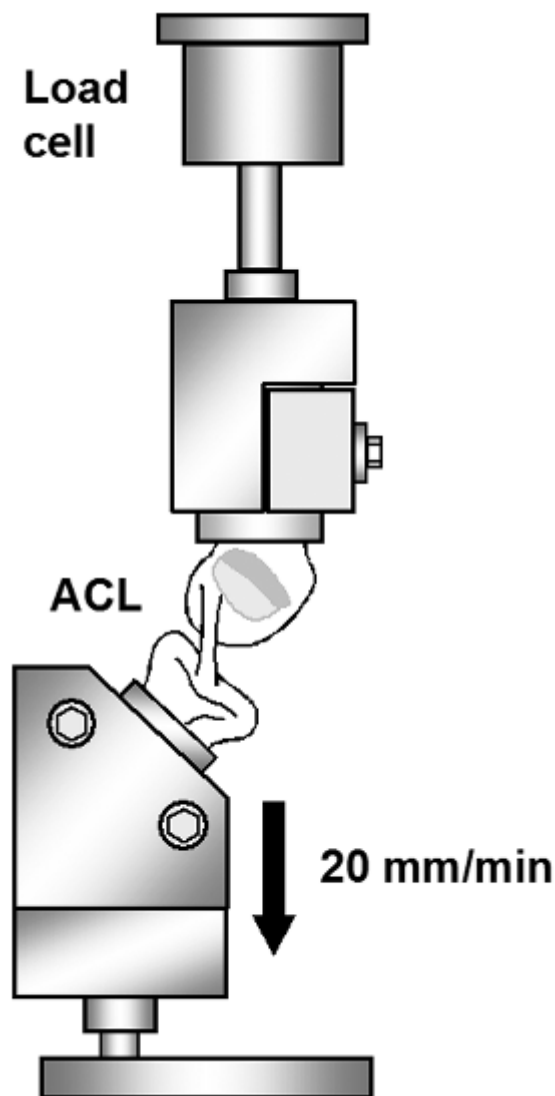
At the time of sacrifice, the lower extremities of each animal were then disarticulated at the hip joint. Each specimen was stored at -80°C until the time of testing. Before mechanical testing, each knee was thawed overnight at 4°C.

The anteroposterior translation of the knee was determined as follows. The femur-knee-tibia complex (45 mm long femur and 60 mm long tibia) was removed from the hindlimb. All the surrounding muscles were carefully dissected. Care was taken to avoid injuring the joint capsule and the ligament tissues. The femur and the tibia were separately cast in cylindrical aluminum tubes (25 mm diameter and 30 mm length) using polymethylmethacrylate resin. The specimen was mounted onto a specially designed testing device having 5 degrees of freedom, which was attached to a tensile tester (RTC-1210, Orientec, Oakabe, Japan).<sup>12</sup> Four cycles of anteroposterior shear loads of 10 N were applied to the knee specimen at 30°, 60°, and 90° of knee flexion, respectively. A crosshead speed was set at 20 mm/min. The specimen was kept moistened throughout the test period with a physiologic saline solution spray. Load-elongation curves were drawn with an X-Y recorder (Model 3023, Yokogawa, Tokyo, Japan). The maximum anterior translation in the load-elongation curve was defined as the anterior translation of the knee at each angle of knee flexion.

Next, the joint capsule and all ligaments except for the ACL were carefully dissected in each specimen. The femur

was clamped with the alligator jaw attached to a steel stand, and the tibia was suspended from the femur with the ACL. A weight was attached to the distal end of the tibia so that a 0.5-N load was applied to the ACL. The femur was inclined so that the knee was flexed at 45°. The length of the ACL was measured with a vernier caliper (Mitutoyo) at the anterior, posterior, medial, and lateral aspects, respectively. The mean of the 4 length values was defined as the length of the ACL.<sup>11</sup> The cross-sectional area (CSA) of the ACL was measured under the same condition as the optical noncontact method using a change-coupled device (CCD) camera (WV-BD400, Panasonic, Osaka, Japan) and a video dimension analyzer (HTV-C1170, Hamamatsu Photonics, Tokyo, Japan), as reported by Yamamoto et al.<sup>36</sup> Briefly, the medial femoral condyle and a portion of the lateral femoral condyle distal to the ACL insertion were resected for visualization with the video dimension analyzer. The femur was attached to the stepping motor, and a constant tensile load of 0.5 N was applied to the ACL by suspending a weight to the tibia. The femur was rotated with the stepping motor at 5° angular increments through 360°, and the corresponding profile width of the ACL was recorded with the video dimension analyzer. The cross-sectional shape of the ACL was reconstructed using a computer algorithm. The measurement was done at the middle of the ACL to quantify a part of gross observation on the thickness of the ACL. Namely, we did not use the CSA to determine the material properties of the injured ACL, but we determined the structural properties of the ACL because there were some problems determining the material properties of the healed ACL in the partial injury model. In addition, the middle (elongated) portion was torn in the postoperative tensile testing. Subsequently, the critically important site in this study is not the transected portions but the elongated portion.

For tensile testing, the femur-ACL-tibia (FAT) complex specimen was mounted onto a tensile tester (PTM-250W, Orientec, Tokyo, Japan) with the use of a set of specially designed grips (Figure 2). The tibia was flexed at 45° against the femur. The knee was rotated approximately 90° toward the internal direction to remove the normal distortion of the ACL,<sup>32</sup> although the loads were not completely applied to all portions of the bundle during tensile testing. Synovium-like tissues that enveloped the injured ACL were not removed for tensile testing or before CSA measurements because we did not intend to determine the material properties of the healed ACL but intended to determine the structural properties. In addition, we measured the apparent CSA to support the gross observations of the thickness of the healed ACL. Before the tensile test, the specimen was preconditioned with a static preload of 0.5 N for 5 minutes, followed by 10 cycles of loading and unloading (3% strain) with a crosshead speed of 20 mm/min. Then, each specimen was stretched to failure at a crosshead speed of 20 mm/min. This speed was chosen because it was frequently used in previous studies with the rabbit ACL.<sup>2,22,26,31</sup> Danto and Woo<sup>5</sup> reported that the effect of the rate of loading on the ACL is relatively small. The specimens were kept moist with physiologic saline solution during testing.



**Figure 2.** The experimental apparatus used to evaluate the structural properties of the femur-ACL-tibia complex.

The load-elongation curve was obtained in the tensile test. The structural properties (maximum load, stiffness, elongation at failure) were determined directly from the load-elongation curves. The maximum load was defined as the load at the point of failure of the specimen. The stiffness was defined by the slope of the load-elongation curve, which was determined by applying a least squares linear regression analysis to the data curve between the endpoint of the toe region and the point starting to bend before failure.

#### Histological Observations

In each limb intended for histological observation, the FAT complex was resected and fixed in a buffered 10% formalin solution, decalcified, and cast in paraffin blocks. For each block, we set a microtome so that the midsubstance was sectioned parallel to the longitudinal axis. The central por-

tion of the whole specimen in the sagittal plane was marked on the block surface. The half of the specimen was resected off until the central sagittal plane could be observed. Then, five 5- $\mu$ m continuous sections were obtained, and they were stained with hematoxylin and eosin for histological observations. Two of the coauthors (K.Y., H.T.) observed the histologic evaluation, independently, under a blinded manner. Cellularity, shape of the nucleus of cells, and collagen striations in the ligament substance were observed with light microscopy.

#### Statistical Analyses

All data were shown as the mean with the standard deviation value. Concerning each parameter, the 1-way analysis of variance (ANOVA) was performed among the groups. When a significant effect was obtained, a post hoc test with the Fisher protected least significant difference test was made for multiple comparisons. A commercially available software program (Stat View, Abacus Concepts Inc, Berkeley, Calif) was used for statistical calculation. The significance level was set at  $P = .05$ .

#### RESULTS

##### Anterior Translation of the Knee

Concerning the anterior translation of the knee (Table 1), the ANOVA showed significant differences among the groups at each angle of knee flexion ( $P < .0006$ ). The post hoc test indicated that, at 30° of knee flexion, groups 1, 2, 3, and 4 were significantly greater than the control group, whereas there were no significant differences among the 4 injured groups.

##### Gross Observation of Inside the Knee Joint

In the 4 injured groups, the ACL appeared to be slack in the knee. In groups 1 and 2, the ACL was enveloped by thin synovium-like tissues at 12 weeks. In these 2 groups, the transected portions could be distinguished from the other portions in the midsubstance. In groups 3 and 4, the ACL was enveloped by thick synovium-like tissues at 12 weeks. In these groups, the transected portions could not be distinguished from the other portions because of the thick synovium-like tissues.

In these 4 injured groups, the infrapatellar fat pad was commonly fibrotic. Either a meniscus tear or a tibiofemoral osteophyte formation was found in the injured groups. The articular cartilage surface partly showed mild degeneration, such as softening or fibrillation, in the injured groups.

##### Tissue Dimension of the ACL

The ANOVA showed a significant difference ( $P = .0467$ ) in the length of the ACL among the groups (Table 1). The post hoc test indicated that the ACL length of groups 1, 2, 3, and 4 was significantly greater than that of the control

**TABLE 1**  
Anterior Translation of the Knee and the Tissue Dimensions of the Anterior Cruciate Ligament (means, with SDs in parentheses)

Group	Anterior Translation of the Knee, mm			ACL Length, mm	Cross-Sectional Area, mm <sup>2</sup>
	30°	60°	90°		
Control	1.1 (0.3)	0.9 (0.2)	0.6 (0.2)	10.2 (0.6)	5.7 (1.3)
Group 1	2.0 (0.3) <sup>a</sup>	1.8 (0.5) <sup>a</sup>	1.4 (0.4) <sup>a</sup>	11.5 (0.6) <sup>a</sup>	5.0 (1.8)
Group 2	2.1 (0.6) <sup>a</sup>	1.8 (0.4) <sup>a</sup>	1.3 (0.5) <sup>a</sup>	11.2 (0.6) <sup>a</sup>	5.7 (2.6)
Group 3	1.9 (0.4) <sup>a</sup>	1.8 (0.5) <sup>a</sup>	1.4 (0.5) <sup>a</sup>	11.4 (1.4) <sup>a</sup>	6.0 (1.1)
Group 4	2.0 (0.4) <sup>a</sup>	2.1 (0.5) <sup>a</sup>	1.5 (0.5) <sup>a</sup>	11.4 (0.6) <sup>a</sup>	4.4 (0.7)

<sup>a</sup>Significantly different from the control group ( $P < .05$ ).

**TABLE 2**  
Absolute Values of the Structural Properties of the Femur-ACL-Tibia Complex (means, with SDs in parentheses)

Group	Maximum Load, N	Stiffness, N/mm	Elongation at Failure, mm
Control	361.8 (51.8)	136.4 (23.6)	3.1 (0.5)
Group 1	174.9 (60.8) <sup>a</sup>	71.7 (22.9) <sup>a</sup>	2.7 (0.5)
Group 2	147.8 (78.5) <sup>a</sup>	65.1 (32.8) <sup>a</sup>	2.9 (0.5)
Group 3	241.8 (46.9) <sup>a-d</sup>	102.1 (10.5) <sup>a-c</sup>	2.8 (0.3)
Group 4	159.6 (34.9) <sup>a</sup>	80.0 (13.6) <sup>a</sup>	2.7 (0.4)

<sup>a</sup>Significantly different from the control group ( $P < .05$ ).

<sup>b</sup>Significantly different from group 1 ( $P < .05$ ).

<sup>c</sup>Significantly different from group 2 ( $P < .05$ ).

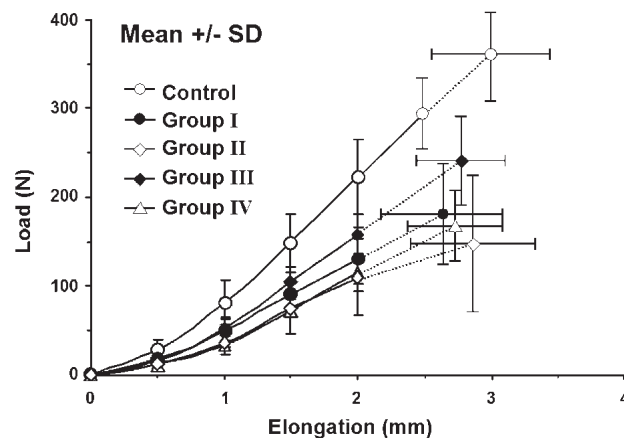
<sup>d</sup>Significantly different from group 4 ( $P < .05$ ).

group, whereas there were no significant differences among the 4 injured groups. Namely, the ACL length of groups 1, 2, 3, and 4 was elongated by approximately 1 mm (approximately 10% of the original length of the whole ACL). Regarding the whole CSA, the ANOVA indicated no significant differences among the groups (Table 1).

**Biomechanical Properties of the FAT Complex**

In tensile testing, failure modes showed that the ACL attachment was avulsed in all specimens in the control group and 6 of the 7 specimens in group 3, whereas almost all specimens failed at the midsubstance in groups 1, 2, and 4 (6, 6, and 5 of 7 specimens, respectively). The load-elongation curves indicated that obvious differences were observed between the control group and the other 4 injured groups (Figure 3).

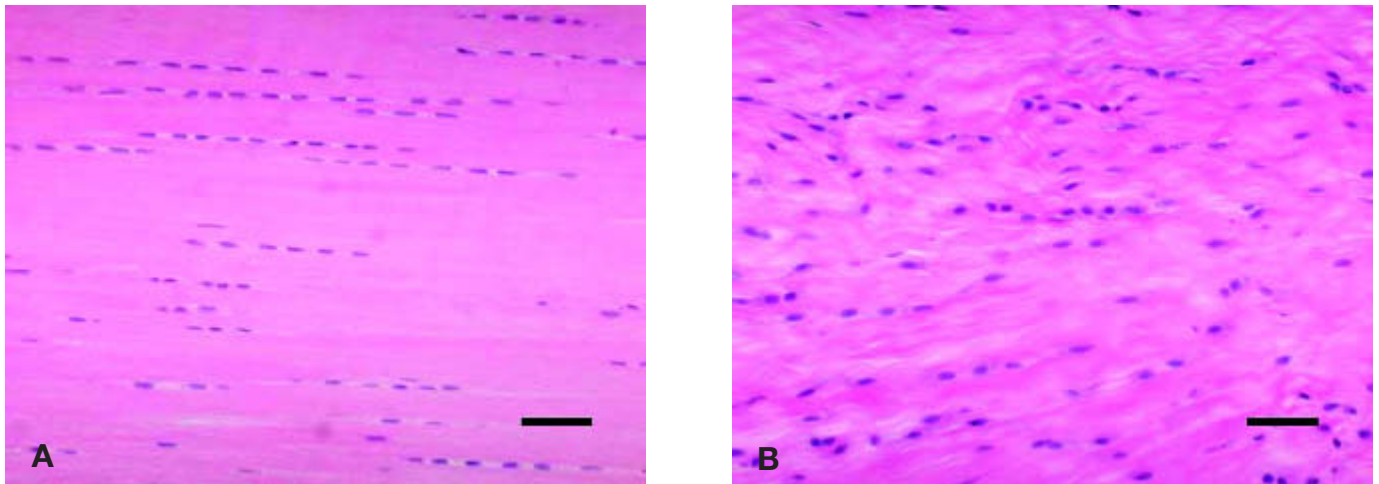
The average maximum load of the FAT complex was 174.9, 147.8, 241.8, 159.6, and 361.8 N in groups 1, 2, 3, 4, and the control group, respectively (Table 2). The ANOVA demonstrated a significant difference ( $P < .0001$ ) in the maximum load among the groups. The post hoc test showed that the maximum load of group 3 was significantly greater than that of groups 1 ( $P = .0345$ ), 2 ( $P = .0041$ ), and 4 ( $P = .0107$ ), respectively, but significantly lower ( $P = .0004$ ) than that of the control group. There were no significant differences in the maximum load among groups 1 and 2. On the other hand, group 4 did not



**Figure 3.** Load-elongation curves for the femur-ACL-tibia complexes. Each error bar represents the standard deviation.

show any significant differences in the maximum load in comparison with groups 1 and 2.

The average stiffness of the FAT complex was 71.7, 65.1, 102.1, 80.0, and 136.4 N/mm in groups 1, 2, 3, 4, and the control group, respectively (Table 2). The ANOVA showed a significant difference ( $P < .0001$ ) in the stiffness among all the groups. The post hoc test showed that the stiffness of group 3 was significantly greater than that of groups 1 ( $P = .0154$ ) and 2 ( $P = .0039$ ) but significantly lower ( $P =$



**Figure 4.** Histological findings in the midsubstance of the ACL with light microscopy. The bar indicates 50  $\mu\text{m}$  (original magnification  $\times 100$ ). A, histology of the normal ligament; B, histology of group 3. In the midsubstance, we occasionally observed granulation-like tissues, in which collagen bundles appeared to be irregular, separated, and fragmented, with numerous cells having a small round nucleus in the treated groups.

.0069) than that of the control group. There were no significant differences in stiffness between groups 1 and 2. On the other hand, group 4 did not show any significant differences in stiffness in comparison with groups 1 and 2.

Regarding the elongation at failure of the FAT complex, the ANOVA revealed no significant differences among the 5 groups (Table 2).

### Histology

In the control group, the normal ligament was covered by a thin synovial membrane. The midsubstance consisted of closely packed collagen fibers, which were aligned longitudinally with a periodic crimp pattern. Fibroblasts were sparsely scattered between the collagen fibers (Figure 4A). In each injured group, the ligaments were covered by relatively thick synovial tissues, and the gap portions, which were sharply cut, were not completely filled with the granulation-like tissues. In the elongated portion of the midsubstance, we occasionally observed granulation-like tissues, in which collagen bundles appeared to be irregular, separated, and fragmented with numerous cells having a small round nucleus (Figure 4B). We could not find any obvious differences among the injured groups, although we could not make statistical comparisons.

### DISCUSSION

In this study, the newly developed ACL injury model was used. A few models for the partial laceration-type ACL injury had been reported in the literature.<sup>8,19,21,30</sup> For example, O'Donoghue et al<sup>21</sup> reported a partial rupture model created by sharply cutting the lateral one half of the tibial insertion. Hefti et al<sup>8</sup> devised a partial rupture model made by cutting the lateral three fourths of the midsubstance. In these models, however, collagen fibers that were not lacerated in the substance remained thoroughly

intact, or, subsequently, the ACL was not elongated at all so that the knee did not show any instability. Therefore, we developed a new elongation-type ACL injury model. In the previous study,<sup>12</sup> we evaluated the state of the ligament in this type of model for 24 weeks after the treatment. Briefly, first, the ACL length was significantly elongated by approximately 10% of the original length throughout the 24-week period. Second, the maximum load and the stiffness of the FAT complex were drastically reduced to approximately 30% of the normal value immediately after surgery and then were gradually increased to 40% to 50% by 12 weeks. Third, the reproducibility of this model was high enough to study the effects of various therapeutic treatments on the injured ACL.

Using this valuable model, this study clearly demonstrated that the application of 4 ng TGF- $\beta$ 1 significantly increases the stiffness of the injured ACL midsubstance. In addition, the results of the failure modes and the maximum load of the FAT complex suggested that this application significantly increases the maximum load of the injured ACL midsubstance. The results implied that TGF- $\beta$ 1 significantly enhances healing in the ACL. However, this study showed that TGF- $\beta$ 1 did not significantly affect the anterior translation of the knee or the ACL length; results suggested that TGF- $\beta$ 1 does not restore the elongated ACL back to the normal length. In addition, the present study implied that the application of 20  $\mu\text{g}$  PDGF-BB does not significantly affect the structural properties of the elongation-type ACL injury.

We can speculate on a few possible mechanisms concerning the in vivo effect of TGF- $\beta$ 1 on the structural properties of the injured ACL. Spindler et al<sup>25</sup> reported that fibroblasts within ACL remnants overexpress type I collagen mRNA after injury. The authors showed that tissue healing is incomplete but definitely does occur in the ACL midsubstance with the elongation-type injury.<sup>12</sup> TGF- $\beta$ 1 is considered to affect the fibroblasts within the injured ACL.

Marui et al<sup>16</sup> reported that TGF- $\beta$ 1 increases not only collagen synthesis but also noncollagenous protein synthesis in ACL fibroblasts. Mauviel<sup>17</sup> and Uria et al<sup>28</sup> demonstrated that TGF- $\beta$ 1 suppresses collagenase activities produced by fibroblasts. Recently, Tohyama et al<sup>26,27</sup> reported that TGF- $\beta$ 1 significantly enhances type I procollagen mRNA expression to a greater degree than type 3 procollagen mRNA expression in fibroblasts. They also reported that TGF- $\beta$ 1 significantly reduces interstitial collagenase mRNA expression in the same cells. Therefore, the application of TGF- $\beta$ 1 may have affected the type I collagen network and/or minor collagen network in the injured ACL and may have reduced the collagenase activity produced by fibroblasts in the injured ACL. In addition, the application of TGF- $\beta$ 1 may affect the collagen cross-link profile. Further studies should be conducted to clarify collagenase activity within the ACL, as well as collagen network or cross-link profile.

In this study, we did not detect any differences as to the histological findings among the injured groups. In general, the injured lesion and the uninjured midsubstance were clearly distinguished in the complete rupture model. In the elongated injury model used in the present study, however, the minimal rupture portions of collagen fibers or fascicles were scattered in the apparently intact midsubstance so that it is difficult to detect the collagen microruptures themselves using a microscope.<sup>12</sup> Therefore, the specific healing reaction by fibroblasts with or without the growth factor treatment was also difficult to precisely observe with a microscope. We consider that this may be the reason why we did not histologically detect any differences in the present study. This may be one of the characteristics in the overstretched model because we found some histological differences when we used the in situ frozen-thawed ACL model in our previous studies.<sup>2,22,26</sup> In future studies, the effect of the application of TGF- $\beta$ 1 may be detected by molecular-biological examinations. However, this study demonstrated the biomechanical evidences in the healing tissues using the same tensile tests as used in the previous studies with the in situ frozen-thawed ACL model.<sup>2,22,26</sup> We believe that it is of value to report these important biomechanical differences in the new overstretched model.

It is important to compare this study with previous studies on MCL injury. Hildebrand et al<sup>9</sup> reported that an application of 20  $\mu$ g PDGF-BB significantly enhances healing of complete MCL injury in a rabbit model, but an application of 4 ng TGF- $\beta$ 1 showed a tendency for an enhancing effect. These in vivo effects of the 2 growth factors in their study were remarkably different from those in our present study. Ultrastructural, histological, biochemical, and biomechanical differences have been reported between the ACL and the MCL tissues with strikingly different healing capacities.<sup>1,15,18,32</sup> In addition, our previous studies<sup>12</sup> showed that healing of the elongation-type ACL injury is greatly different from that of the complete injury of the MCL.<sup>29,33</sup> Therefore, these results implied that there is the strong possibility that in vivo effects of the 2 growth factors may be different between ACL and MCL injuries. In addition, Tohyama et al<sup>26,27</sup> reported that PDGF-BB

significantly enhances interstitial collagenase mRNA expression in fibroblasts. These results partly explain why PDGF-BB did not significantly affect the ACL injury in the present study. Thus, the difference between the present study and the study by Hildebrand et al<sup>9</sup> implied that the in vivo effect of growth factors on ligament injuries may be different between intra-articular and extra-articular environments or between the ACL and the MCL.

There are some limitations in the present study. The first limitation is that we did not perform elution studies by ourselves to demonstrate how long the growth factors would be present. However, Giannoni and Hunziker<sup>7</sup> reported an in vitro elution study wherein the elution of the factors from a fibrin sealant was measured over time. They demonstrated that the liberation of [<sup>125</sup>I]-labeled TGF- $\beta$ 1 from fibrin matrix was monitored by liquid scintillation counting for 25 days in vitro. During the initial 5 days, fibrin clots containing TGF- $\beta$ 1 released this cytokine at 10% to 20% per day. After 10 days of incubation, 40% to 50% of the TGF- $\beta$ 1 was present within the fibrin matrix. At the end of the incubation period, 68% of the TGF- $\beta$ 1 had been released from the fibrin clots. We believe that in the present study there was the similar condition on the elution of the factors from a fibrin sealant. In addition, we have used the same type of fibrin sealant as a vehicle for TGF- $\beta$ 1 and PDGF-BB in the in vivo studies, and the positive effect of the growth factors was proven not only on the in situ frozen-thawed ACL<sup>2,22,26</sup> but also on the bone-patellar tendon-bone graft.<sup>37</sup> Therefore, the fibrin sealant was considered to act as a useful vehicle releasing these growth factors in the in vivo conditions. Second, although Coffey et al<sup>4</sup> demonstrated that systemic clearance of the active form of TGF- $\beta$ 1 is rapid, it has been reported that the in vivo application of some growth factors including TGF- $\beta$ 1 chronically enhances tissue healing and remodeling.<sup>2,22,26,34,35,37</sup> Although the mechanisms are not yet known, this study showed that the in vivo application of TGF- $\beta$ 1 significantly affects healing of the injured ACL.

The second limitation is that the injury created in the ACL in this study is not the same as human ACL injuries with partial laceration and permanent elongation, specifically concerning the artificial transection portions. However, human ACL midsubstance injuries with various degrees of overstretched portions and lacerated portions actually occur in athletic accidents,<sup>20</sup> and the present ACL injury model has an ACL midsubstance injury with an almost constant degree of overstretched portions and lacerated portions. Therefore, we believe that this model, having the overstretched portions and the lacerated portions, can be a model of the elongation-type ACL injury in human patients. In addition, interestingly, the elongated midsubstance portion was weaker than the transected portion in the postoperative tensile testing. Therefore, we can say that the elongated portion was critically more important than the transected portion. Commonly speaking, we have no ideal partial or complete ligament injury model that is perfectly similar to human ligament injuries. All the ACL injury models reported in the literature<sup>8,19,21,30</sup> were created by partially or completely cutting the midsubstance. We believe that the present ACL injury model

is not ideal but an acceptable model compared to the previous models.

The third limitation is that we did not quantify the force that induced irreversible lengthening of the ligament; therefore, we did not know the magnitude of this force. However, we controlled this procedure by quantification of the length. The fourth limitation is that we did not measure the half-life time of TGF- $\beta$ 1 and PDGF-BB in this application into the joint cavity. The fifth limitation is that in the present study, we could not clarify when the effect of each growth factor occurred during the experimental period. The sixth limitation is that we could not quantify histological findings. Beyond these limitations, however, we believe that this study provides valuable information on the effect of the 2 growth factors on this elongation-type ACL injury model.

It is hard to directly infer clinical relevance of this study because this is an experimental study. At the present time, however, it is extremely important to increase the database concerning in vivo effects of growth factors on ligament injuries for future applications. From this viewpoint, this information is considered to be valuable for future therapeutic application of growth factors to ligament injuries.

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