Is the increase in type III collagen of the patellar tendon graft after ligament reconstruction really caused by "ligamentization" of the graft?

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Abstract: To test the hypothesis that extrinsic cells that infiltrate the devitalized patellar tendon (PT) synthesize type III collagen even in the environmental milieu of the native PT, we conducted the present experimental study using the rat *in situ* frozen-thawed PTs. Tissue culture showed no cell outgrowth from the tendons after the freeze-thaw treatment. Analysis by RT-PCR showed that the expression level of type III procollagen mRNA in the frozen-thawed tendon was significantly higher than that in the sham-operated tendon at 6 and 12 weeks. Immunohistological findings showed positive type III collagen staining around cells that had infiltrated the necrotized tendon at 3, 6, and 12 weeks. In addition, the elastic modulus of the *in situ* frozen-thawed tendon at 6 weeks was significantly less than that of the sham-operated tendon. The present study indicates that extrinsic cells that had infiltrated the devitalized PT synthesized type III collagen at least for 12 weeks even in the environmental milieu of the native PT. These findings raised the question of whether the increase in type III collagen of the PT graft after ACL reconstruction is really caused by "ligamentization," the adaptation of the PT graft to the ACL environment.

The fate of trasplanted patellar tendons (PTs) for reconstruction of the anterior cruciate ligament (ACL) has been studied extensively [1,2,3,11]. These studies have shown that the PT graft to reconstruct the ACL undergoes dramatic changes in its structure. These changes can be divided into four stages: (i) avascular necrosis, (ii) revascularization, (iii) cellular proliferation, and (iv) remodeling. In this process, PT grafts showed a gradual assumption of the biochemical characteristics of the normal ACL. For example, Amiel et al. [2] reported that a gradual increase in the concentration of type III collagen was seen in the PT grafts; by 30 weeks the concentration (10%) was the same as in normal ACL; type III collagen was not observed in the native PT. Their results suggest that PT grafts undergo a process of "ligamentization" when they are placed in an ACL environment.

Despite a number of experimental studies involving ACL reconstruction, it remains unknown whether the changes in the matrix of the PT graft after ACL reconstruction are caused by the adaptation of the PT to the ACL environment. We hypothesized that extrinsic cells that infiltrate the devitalized PT synthesize and secrete type III collagen even in the environmental milieu of the native PT. To simulate tendon graft for ligament reconstruction, several techniques for the *in situ* freeze-thaw treatment of tendons and ligaments have been developed [5,7,8,9,10,15,16,17,20,21]. These techniques devitalize intrinsic fibroblasts in the tissues without surgically disturbing the anatomical orientation, the anatomical attachments, or the physiological tension of the tissues. The purpose of the present study is to test the hypothesis that extrinsic cells that infiltrate the rat PT after the *in situ* freeze/thaw treatment synthesize type III collagen even in the environmental milieu of the native PT using the reverse transcription-polymerase chain reaction (RT-PCR) assay, immunohistology, and

mechanical evaluation.

MATERIALS AND METHODS

Animals

This study involved 65 male 16-week-old Wistar-King rats weighing 360 ± 20 g (mean ± SD). All animal procedures were carried out under the rules and regulations of the animal care and use committee in the authors' institution.

Experimental Design

The *in situ* freeze-thaw treatment was performed on the right PTs of 50 rats, while the left PTs of these rats (sham-operated tendons) underwent sham operations. These rats were used for RT-PCR analysis (n=15), immunohistochemical observations (n=20), and mechanical evaluation (n=15). Additionally, the right non-treated normal PTs of 10 rats served as the controls for RT-PCR analysis (n=5) and the immunohistological observations (n=5). Ten PTs of five rats were used for tissue culture to evaluate the effect of the *in situ* freeze-thaw treatment on the viability of intrinsic fibroblasts in patellar tendons.

Surgical Procedures

All animals were operated on under sterile conditions with pentobarbital (50 mg/kg, i.p.). After the posterior surface of the right patellar tendon was separated from the infrapatellar fat pad, a silicone rubber sheet (20 mm length x 15 mm width x 1.0 mm thickness) was inserted between the patellar tendon and the fat pad to make a contained

field with the tendon inside it (Fig. 1). The contained field was then filled with liquid nitrogen. The patellar tendon was frozen for 1 min. The frozen patellar tendon was then thawed by physiological saline solution poured into the contained field. In the left knee, the patellar tendon underwent the same surgical treatment except that the tendon was not frozen with liquid nitrogen. No immobilization was applied postoperatively, and each animal was allowed unrestricted activity in the cage.

Tissue Culture

To confirm that *in situ* freeze-thaw treatment necrotizes fibroblasts in the rat patellar tendon, tissue cultures from five animals were grown. Immediately after the *in situ* freeze-thaw treatment, the right patellar tendon was surgically excised and carefully dissected from the surrounding connective tissue. The normal left patellar tendon was also obtained in the same manner. Each excised tendon was divided into 1-mm^2 pieces (25-30 depending on the size of the tendon). Twenty randomly chosen explants from each tendon were placed into culture dishes, and each explant was attached to the bottom of the dish with 10 µl of 0.2% calf skin type I collagen gel (Koken, Tokyo, Japan). Finally, the culture dishes were flooded with Dulbecco's modified Eagles' medium containing 10% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic (penicillin G, 100,000 U/ml; streptomycin sulfate, 10,000 µg/ml; and fungizone, 25 µg/ml). The cultures were maintained at 37°C in a humidified incubator containing a mixture of 5% CO_2 in air. After 21 days of incubation, the number of explants with fibroblast outgrowth in each culture dish was counted using a phase-contrast microscope.

RT-PCR

We performed RT-PCR analysis to evaluate the expression of type III procollagen mRNA of the patellar tendon after the *in situ* freeze-thaw treatment. At 3, 6, and 12 weeks after surgery, five animals per time point were anesthetized and perfused transcardially with 100 ml of saline to eliminate blood from the patellar tendons. In addition, four normal right patellar tendons (controls) were obtained in the same manner. Immediately after each animal's death, the tendons were excised, stripped of excess connective tissue, and immediately frozen in liquid nitrogen. Total RNA was extracted from the tissue with the Isogen™ RNA extraction kit (Nippon Gene, Tokyo, Japan). A 2-µg aliquot of total RNA was reverse-transcribed with a first-strand cDNA synthesis kit (Amersham Pharmacia Biothech, Piscataway, NJ, USA) using pd(N)6 primer. The 2-µl aliquots of the 33-µl reverse-transcription samples were subjected to amplification by the polymerase chain reaction (PCR) method for 25 cycles with denaturation at 94°C for 1 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min using 0.625 U of EX *Taq* polymerase (Takara Biomedical, Shiga, Japan). Type III procollagen primers were 5'-GTGCTGCCATTGCTGGAGTT-3' (900-919) (forward) and 5'-CCGGCTGGAAAGAAGTCTGAG-3' (1384-1364) (reverse). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers used were 5'-TGAAGGTCGGTGTCAACGGATTTGGC-3' (35-60) (forward) and 5'-CATGTAGGCCATGAGGTCCACCAC-3' (1017-994) (reverse). After amplification, the products were analyzed by 2% agarose gel electrophoresis with ethidium bromide, and signals were digitized and quantified by an MCID image analyzer (Imaging Research Inc., St. Catharine’s, Ontario, Canada). Background values were determined from an equivalent surface area near each signal and subtracted

from each value before normalization. Integrated density values from the frozen-thawed and the sham-operated tendons were normalized to the value of the control tendon in the same gel. Then, the normalized value of type III collagen expression relative to that of the internal control, GAPDH, was analyzed. For each sample, we also carried out 30 cycles of PCR for GAPDH and confirmed that PCR for 25 cycles did not reach a plateau.

Immunohistochemistry

At 2, 3, 6, and 12 weeks after surgery, five animals per time point were anesthetized and perfused transcardially with 50 ml of 10% neutral buffered formalin. Then, the frozen-thawed and the sham-operated tendons were excised. In addition, five normal patellar tendons were obtained in the same manner from the right knees of animals that did not undergo surgery. After fixation, the specimens were embedded in paraffin, and longitudinal sections, 7-μm thick, were sliced with a microtome and fixed onto glass slides. The sections were stained for type III collagen with the streptavidin-biotin-peroxidase complex procedure using a commercial kit (Histofine™ SAB-PO Kit; Nichirei, Tokyo, Japan) according to the manufacturer's protocol. We used mouse anti-rat type III collagen monoclonal antibody at a dilution of 1:100 (Quartett, Berlin, Germany) as the primary antibody. The sections were counterstained with Mayer's hematoxylin.

Mechanical Testing

At 0, 3, and 6 weeks after surgery, five animals per time point were anesthetized and perfused transcardially with 100 ml of saline. The limb specimens

were stored in airtight bags at –32ºC until mechanical testing was performed. Before testing, each specimen was thawed overnight at 4°C [25]. The patella-patellar tendon-tibia complex was carefully dissected from each limb. Using an area micrometer, we measured the cross-sectional area of each patellar tendon at three different positions (the middle of the patellar tendon and positions 1 mm proximal and 1 mm distal to the middle). The area micrometer has a 3-mm wide and 3-mm thick rectangular slot and a plunger that inserts into the slot. The patellar tendon was placed in the slot, and the plunger was inserted and pressed against the tendon with a constant compressive stress of 0.12 MPa applied for a 2-minute period [13,22,26]. This stress compressed the tendon to fill the slot while a micrometer was used to measure the thickness of the tendon. The cross-sectional area of the patellar tendon was calculated by multiplying the slot width by the measured thickness. The cross-sectional area was represented by an average of three values obtained from the three different positions. After the measurement of the cross-sectional area, the bone-tendon preparation was mounted on a set of specially designed grips and attached to a tensile tester with the long axis of the tibia flexed at a 45° angle to that of the patella (Fig. 3). The specimen was then stretched in 37°C physiological saline solution at a rate of 20 mm/min to the point of tendon failure. The strain on the tendon was measured by a video dimension-analyzer system during tensile testing. From a stress-strain curve, the elastic modulus of the tendon, the slope of the linear portion in the curve, was determined [22,26].

Statistical Analysis

Unpaired *t*-tests (two-tailed) were carried out on RT-PCR data and mechanical

data for comparison between the frozen-thawed and the sham-operated tendons at each time point. The significance limit was set at p=0.05.

RESULTS

The effect of the *in situ* freeze/thaw treatment on cell viability

No cell outgrowth was observed from explants of the tendons after the freeze-thaw treatment. For the sham-operated patellar tendons, fibroblast outgrowth was observed from 16.6 ± 3.5 per 20 explants, 83.0 ± 17.5 %.

Expression of type III procollagen mRNA

The expected nucleotide size (485 bp) of the RT-PCR transcript was observed, indicating type III procollagen mRNA expression (Fig. 3). The relative intensity of type III procollagen mRNA to GAPDH expression was significantly higher for the frozen-thawed tendon than for the sham-operated tendon at 6 and 12 weeks (Fig. 4) (6 weeks: p=0.016, power=0.768; 12 weeks: p=0.017, power=0.754). No significant differences were found between the frozen-thawed and the sham-operated tendons at 3 weeks (p=0.114, power=0.337).

Distribution of type III collagen

Normal tendons had no staining for type III collagen and uniform cellularity. Two weeks after the freeze-thaw treatment, no cells and no staining for type III collagen were seen in the middle part of the tendon (Fig. 5-A). At 3 weeks, fibroblasts were observed in the anterior and posterior parts of the tendon and staining for type III

collagen was observed in the area with the cellular infiltration. At 6 weeks, intense staining for type III collagen that corresponded with cell distribution was observed (Fig 5-B). At 12 weeks, intense staining for type III collagen was widely distributed throughout the frozen-thawed tendon (Fig. 5-C). For the sham-operated tendon, minimal staining for type III collagen and uniform cellularity were observed throughout the 12 weeks (Fig. 5-D).

Mechanical properties of the patellar tendons

The cross-sectional area of the frozen-thawed tendon was significantly greater than that of the sham-operated tendon at 6 weeks ($p=0.001$, power=1.000), while no significant differences were found between the frozen-thawed and the sham-operated tendons at time-0 or 2 weeks (time-0: $p=0.959$, power=0.050; 2 weeks: $p=0.167$, power=0.258) (Fig. 6-A). During tensile testing, all specimens, except for four frozen-thawed tendons, failed by avulsion fractures at 6 weeks (Table 1). Therefore, we did not analyze the tensile strength of the tendons. The elastic modulus of the frozen-thawed patellar tendon was significantly lower than that of the sham-operated tendon ($p=0.001$, power=1.000), while no significant differences were found between the frozen-thawed and the sham-operated tendons at time-0 or 2 weeks (time-0: $p=0.755$, power=0.059; 2 weeks: $p=0.358$, power=0.134) (Fig. 6-B). The ultimate failure load of the bone-tendon-bone complex at 6 weeks after the freeze-thaw treatment was significantly lower than that of the sham-operated tendon ($p=0.011$, power=0.834) (Fig. 6-C). No significant differences were found between the frozen-thawed and the sham-operated tendons at time-0 or 2 weeks (time-0: $p=0.496$, power=0.095; 2 weeks: $p=0.624$, power=0.073).

DISCUSSION

In the present study, we tested the hypothesis that cells that infiltrate the devitalized PT synthesize type III collagen in the environmental milieu of the native PT. First, the explant culture experiment of the present study showed that the present *in situ* freeze-thaw treatment devitalized intrinsic fibroblasts in the rat PT. Second, RT-PCR analysis in the present study demonstrated that type III procollagen mRNA was over-expressed at the patellar tendon 6 and 12 weeks after the *in situ* freeze-thaw treatment. Third, the immunohistological findings of the present study showed that the expression of type III collagen was observed around fibroblasts that had infiltrated the patellar tendon at 3 weeks or later after the *in situ* freeze-thaw treatment. Therefore, the findings of the present study revealed that cells that had infiltrated the devitalized PT synthesized type III collagen at least 12 weeks even in the environmental milieu of the native PT. In addition, our mechanical evaluation of the present study revealed that the cross-sectional area of the devitalized PT significantly increased and its material properties deteriorated due to a process of the increase of type III collagen in the PT.

A few limitations are apparent in the current study. First, we did not evaluate biochemical parameters of the PT matrix besides the collagen type after the *in situ* freeze/thaw treatment. Amiel et al. [2] reported significant changes in the glycosaminoglycan content and collagen cross-linking of the PT graft after the ACL reconstruction as well as changes in the type of collagen. Therefore, the changes in the glycosaminoglycan content and collagen cross-linking of the PT should also be clarified after the *in situ* freeze-thaw treatment. The second limitation is that we did

not evaluate the tensile strength of the tendon because most of the specimens failed at their insertion sites during mechanical testing.

Several investigators have evaluated the effect of the *in situ* freeze-thaw treatment on cellular viability within several kinds of tendons and ligaments. Jackson et al. [9] developed an *in situ* freeze-thaw model of the ACL with specially designed freezing probe and demonstrated that their procedure killed 93% to 100% of cells within goat ACLs using the cell culture. Bush-Joseph et al. [5] also reported that the same procedure as that by Jackson et al. killed 98% to 94% of cells within goat ACLs. Katsuragi et al. [10] applied a freeze-thaw technique similar to that by Jackson et al. to canine ACLs and showed that their technique killed 98% to 100% of cells within ACLs. We developed an *in situ* freeze-thaw model of rabbit PTs with liquid nitrogen, a technique similar to that of the present study, and showed that this procedure killed 97% to 100% of cells within rabbit PTs using the cell culture [20]. In the present study, we used explant culture to evaluate the effect of *in situ* freeze-thaw treatment on cellular viability within rat PTs due to the small size of rat PTs and verified that our *in situ* freeze-thaw treatment killed practically all cells within rat PTs, although we did not quantitatively know what percentage of cells our *in situ* freeze-thaw procedure killed in rat PTs. Therefore, our *in situ* freeze-thaw technique provided the intrinsic fibroblast necrosis of rat PTs with the preservation of the environmental milieu of native PTs.

In the present study, RT-PCR analysis showed that type III procollagen mRNA was up-regulated in extrinsic cells that infiltrated the PT up to 12 weeks after intrinsic cell necrosis. On the other hand, Sakai et al. [17] reported that type III procollagen gene expression peaked 1 week after the injury and subsequently declined in the healing medial collateral ligament. The difference in type III procollagen gene expression

between these studies suggests that remodeling of the tendon graft is likely slower than that of the injured ligament. In the present study, the level of type III procollagen mRNA at 3 weeks was slightly but not significantly higher in the sham-operated tendon than in the frozen-thawed tendon. Sakai et al. [17] observed in their *in situ* hybridization study an increased expression of type III procollagen mRNA at the ends of a previously normal ligament in an early phase after its injury. Their finding suggests that the inflammatory response to surgical procedures induces up-regulation of type III procollagen mRNA not only in the surrounding tissue but also in native fibroblasts of the ligament. Therefore, in the current study, the inflammatory response of native fibroblasts in the PT to the sham operation might contribute to the up-regulation of type III procollagen mRNA in the sham-operated tendon 3 weeks after surgery.

The present study provided important information on the remodeling process in the PT graft after intrinsic fibroblast necrosis. Previously, we showed that cellular infiltration into the PT stimulated the increase in the cross-sectional area and mechanical deterioration of the patellar tendon after intrinsic fibroblast necrosis [20]. The present study confirmed an increase in the cross-sectional area and a decrease in the elastic modulus in the patellar tendon 6 weeks after the *in situ* freeze-thaw treatment, in which type III collagen was expressed at a higher level than in the sham-operated tendon. The authors previously reported that the number of small-diameter fibrils in the PT significantly increased after the extrinsic cell-repopulating period, using the same model as in the present study [21]. Fleischmajer et al. [6] reported that type III is often codistributed with type I collagen and forms small-diameter fibrils. Therefore, the increase in type III collagen produced by cells of the extrinsic origin may be one of

the mechanisms that induce the increase of small-diameter fibrils and cross-sectional area of the PT as well as mechanical deterioration of the PT after intrinsic fibroblast necrosis.

The fibrillar collagens, such as types I and III, are recognized as the essential building blocks that provide tendons and ligaments with their extraordinary tensile strength and elasticity. The change in collagen fibers is the cornerstone of the graft remodeling after ligament reconstruction [1,4,12,14,18,19]. It is well known that expression of type III collagen in upregulated immediately after tendon healing [23,24]. Type III collagen appears to play a pivotal role in tendon healing. Amiel et al. [2] evaluated collagen typing of the PT graft after rabbit ACL reconstruction. They found that type III collagen was detected in the PT graft as early as 2 weeks after surgery, while the PT had no detectable type III collagen before the implantation. The amount of type III collagen reached a maximum at 6 weeks, and it decreased only slightly by 30 weeks after the operation. They attributed these changes in collagen typing of the PT graft after ACL reconstruction to functional adaptation of cells in that had infiltrated the PT graft from extragraft origin. They called this process 'ligamentization." However, the present study showed that that cells that had infiltrated the devitalized PT synthesized type III collagen at least for 12 weeks even in the environmental milieu of the native PT. These findings raised the question of whether the increase in type III collagen of the PT graft after ACL reconstruction is really caused by "ligamentization." A more thorough mechanism of changes in the graft matrix after ligament reconstruction is critical to the development of a successful means of enhancing tendon graft remodeling after ligament reconstruction. Therefore, a great deal more research should be conducted to clarify the mechanism of changes in the graft matrix after

ligament reconstruction.

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Table 1. Failure modes of the bone-tendon-bone complex for mechanical evaluation

	Tendon failure	Avulsion fracture	
		Patella	Tibia
Time-0			
Frozen-thawed	0	4	1
Sham-operated	0	4	1
2 weeks			
Frozen-thawed	0	3	2
Sham-operated	0	5	0
6 weeks			
Frozen-thawed	4	1	0
Sham-operated	0	4	1

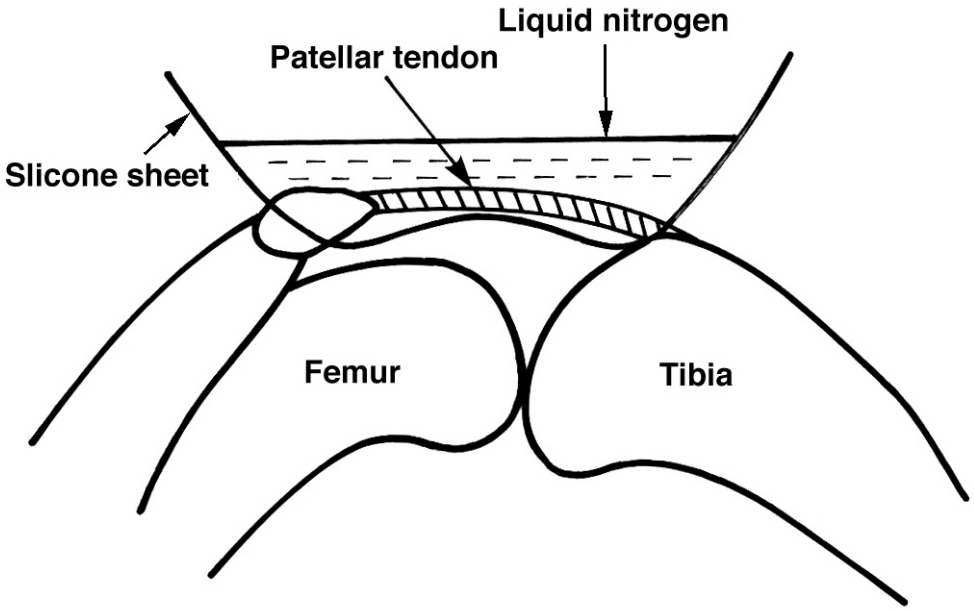

Figure 1. Illustration showing the freeze-thaw technique for the patellar tendon. A silicone rubber sheet is inserted between the patellar tendon and the fat pad to make a contained field with the tendon inside it. The contained field is filled with liquid nitrogen. The frozen patellar tendon is then thawed by physiological saline solution poured into the contained field.

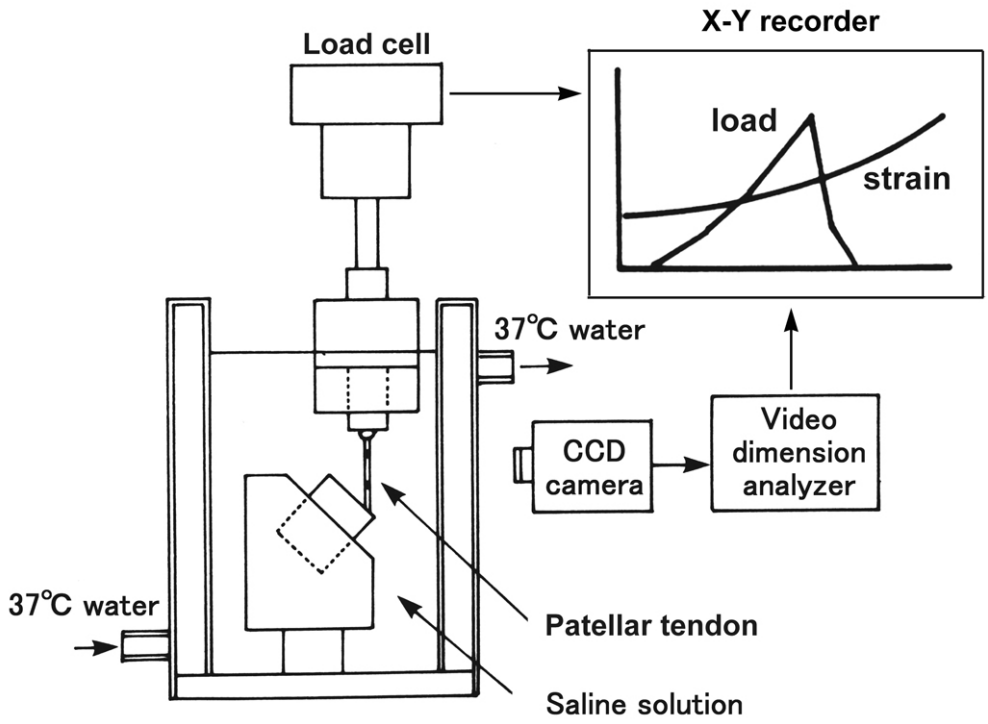

Figure 2. The mechanical testing set-up.

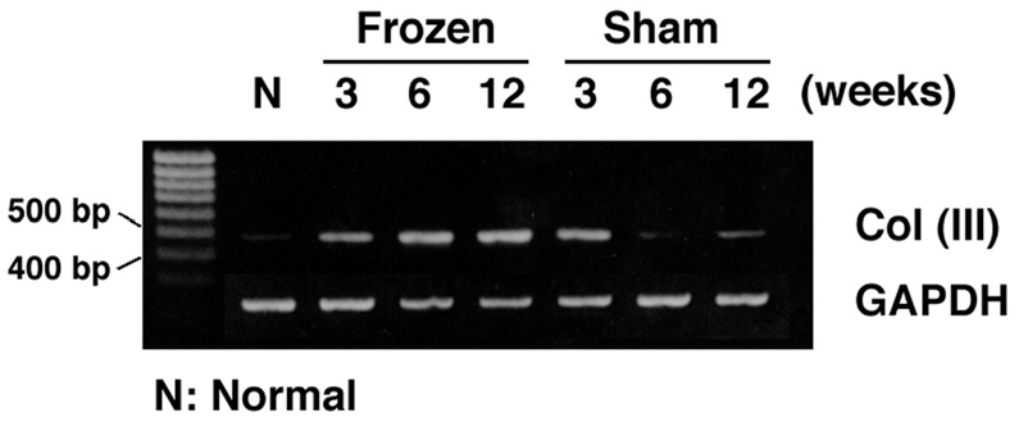

Figure 3. Representative RT-PCR analyses of type III procollagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

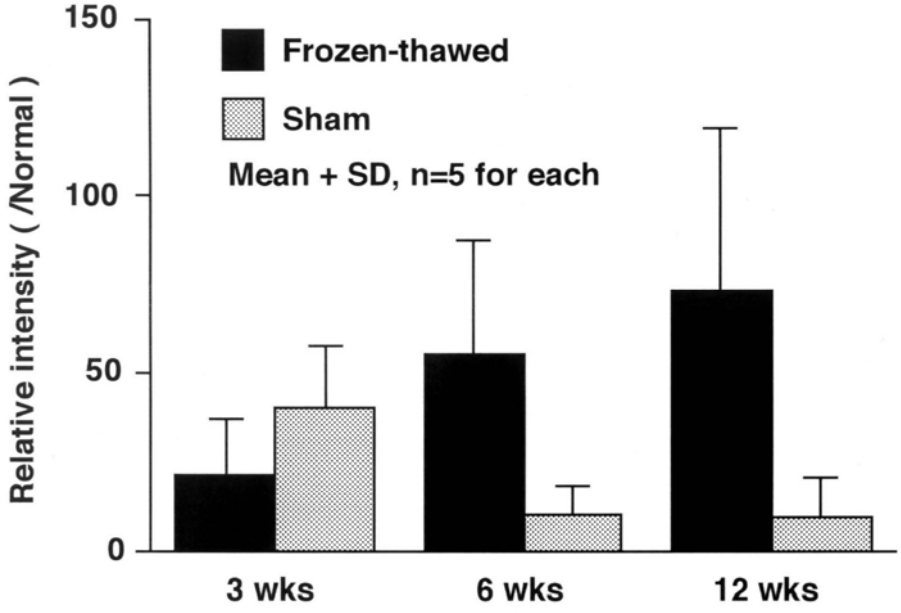

Figure 4. Relative intensity of type III procollagen mRNA to GAPDH expression in RT-PCR analysis.

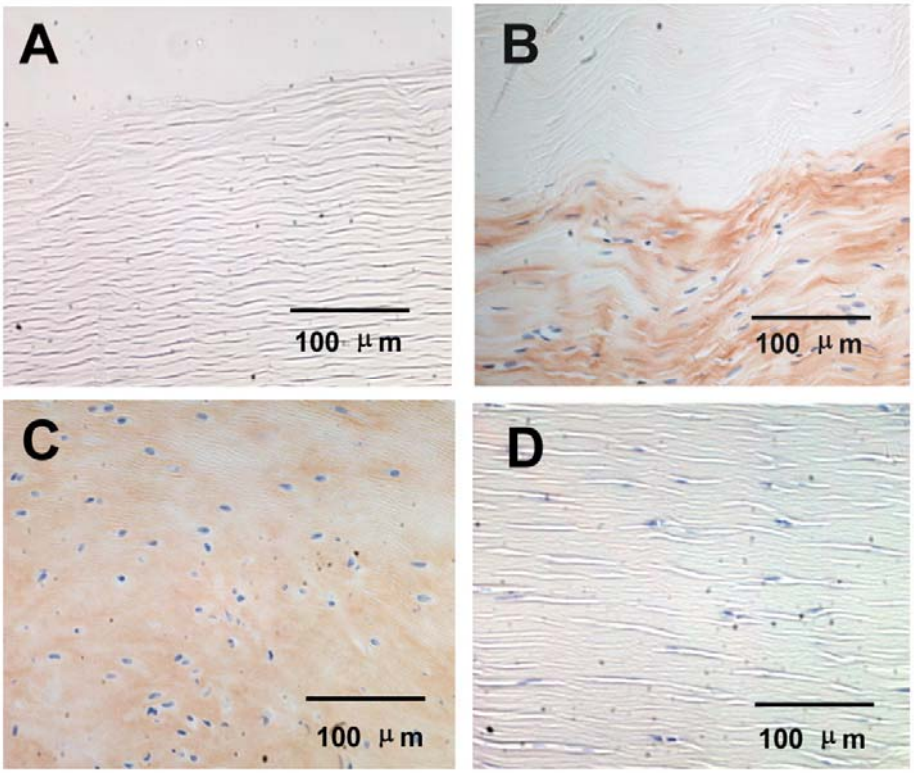

Figure 5. Immunolocalization of type III collagen in the *in situ* frozen-thawed patellar tendons at 2 (A), 6 (B), and 12 weeks (C), and in the sham-operated tendon at 2 weeks (D). Note that type III collagen immunostaining (brown) is observed in the *in situ* frozen-thawed patellar tendons at 6 (B) and 12 (C) weeks, while no immunostaining is seen in the sham-operated tendon (D).

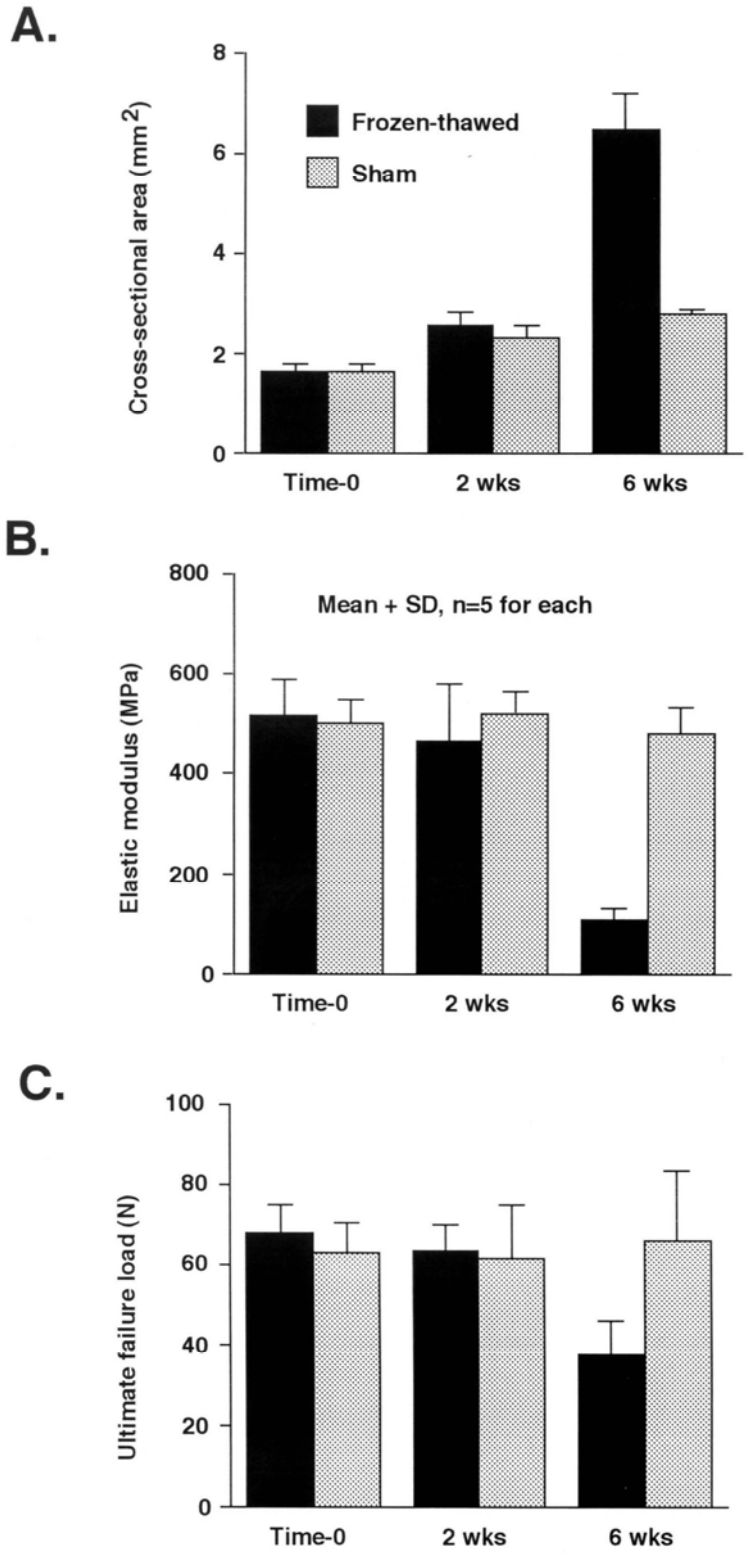

Figure 6. A cross-sectional area (A), the elastic modulus (B) of the patellar tendon, and the ultimate failure load of the bone-patellar tendon-bone complex (C).

Figure legends

Figure 1. Illustration showing the freeze-thaw technique for the patellar tendon. A silicone rubber sheet is inserted between the patellar tendon and the fat pad to make a contained field with the tendon inside it. The contained field is filled with liquid nitrogen. The frozen patellar tendon is then thawed by physiological saline solution poured into the contained field.

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Figure 3. Representative RT-PCR analyses of type III procollagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Figure 4. Relative intensity of type III procollagen mRNA to GAPDH expression in RT-PCR analysis.

Figure 5. Immunolocalization of type III collagen in the *in situ* frozen-thawed patellar tendons at 2 (A), 6 (B), and 12 weeks (C), and in the sham-operated tendon at 2 weeks (D). Note that type III collagen immunostaining (brown) is observed in the *in situ* frozen-thawed patellar tendons at 6 (B) and 12 (C) weeks, while no immunostaining is seen in the sham-operated tendon (D).

Figure 6. A cross-sectional area (A), the elastic modulus (B) of the patellar tendon, and the ultimate failure load of the bone-patellar tendon-bone complex (C).