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Stress shielding of patellar tendon: effect on small-diameter collagen fibrils in a rabbit model

Tokifumi Majima[1], Kazunori Yasuda[2], Takamasa Tsuchida[2], Kunio Tanaka[3], Kiyoshi Miyakawa[3], Akio Minami[1], and Kozaburo Hayashi[4]

[1] Department of Orthopaedic Surgery, Hokkaido University School of Medicine, N-15, W-7, Kita-ku, Sapporo 060-8631, Japan
[2] Department of Medical Bioengineering and Sports Medicine, Hokkaido University School of Medicine, Sapporo, Japan
[3] Central Laboratory for Research and Education, Asahikawa Medical College, Asahikawa, Japan
[4] Division of Bioengineering, Graduate School of Engineering Science, Osaka University, Toyonaka, Japan

Abstract The purpose of this study was to assess the effects of stress shielding on the microstructure and ultrastructure of the patellar tendon using 40 mature female Japanese white rabbits. The patellar tendon was completely released from stress by drawing the patella toward the tibial tubercle with a stainless steel wire installed between them. Microstructurally, stress shielding for 3 and 6 weeks increased the number of cells approximately fivefold, to that of the control tendon. Collagen bundles were less well oriented in the stress-shielded tendon than in the control. Ultrastructurally, small collagen fibrils with a diameter of less than 90 nm increased in the stress-shielded tendon. The median collagen fibril diameter in 6-week stress-shielded tendon was significantly smaller ($P < 0.05$) than in the control tendon (58.8% of control). The ratio of the total area of collagen fibrils to the whole visualized area in the stress-shielded patellar tendon was significantly smaller at 3 and 6 weeks than that in the control. This study demonstrated that complete stress shielding significantly affects the microstructure and ultrastructure of the patellar tendon

Key words Stress shielding · Patellar tendon · Collagen fibril diameter · Ultrastructure

Introduction

Disuse of diarthrodial joints is accompanied not only by many musculoskeletal problems but also by treatments for various disorders, such as immobilization, prohibition of weight-bearing, and bed rest. Stress deprivation has been regarded as the most essential causative factor during joint disuse.[3] Changes in tendons due to joint disuse have been studied mainly using an immobilization model histologically and biomechanically.[1,2,8,9,15,16,20,21] However, it is difficult to control the degree of stress shielding in the immobilization model. In addition, effects of stress deprivation and loss of joint motion cannot be distinguished in the immobilization model. It is necessary to create a model in which the effect of stress deprivation on the tendon tissue can be isolated from the other causative factors in the disused joint.

We have developed such an experimental model using the rabbit patellar tendon.[10,11,17,22,23] In this model, the patellar tendon is slackened without immobilizing the knee joint by drawing the patella toward the tibial tubercle; this position is maintained using a stainless steel wire or a polyester artificial ligament, which is installed between the patella and tibia.

We have conducted a series of studies using this model to clarify biomechanically the effect of stress deprivation on the patellar tendon.[10,11,17,22,23] In these studies, we have clarified that stress deprivation significantly increases the cross-sectional area of the patellar tendon, and that it dramatically reduces the mechanical properties of the tendons. Moreover, the effects were dependent on the degree of stress reduction.[11] No investigators have yet studied the effect of stress deprivation on the ultrastructure of the patellar tendon.

The collagen fiber diameter is directly related to the tensile strength of the skin wound in a guinea pig model.[6] Therefore, we hypothesized that stress deprivation may change the distribution of the collagen fibril diameter: specifically, small diameter of collagen fibrils increases with changes in the number of cells. The purpose of this study was to clarify the effect of complete stress deprivation on the microstructure and ultrastructure of the patellar tendon.

Materials and methods

Experimental design

A total of 40 mature female Japanese white rabbits weighing 3.3 ± 0.2 kg (mean ± SD) were used. The

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rabbits were divided into two groups. In the stress-shielded group ($n = 16$), tension in the right patellar tendon was completely released by pulling a steel wire installed between the patella and the tibial tubercle for a prescribed period.[17,22] The animals of the sham group ($n = 16$) underwent the same surgical procedure, although the wire was not pulled; therefore, the tension in the patellar tendon remained physiological in this group. The remaining eight rabbits were used as normal controls. Animal experimentation was carried out in the Institute of Animal Experimentation, Hokkaido University School of Medicine under the Rules and Regulations of the Animal Care and Use Committee, Hokkaido University School of Medicine.

None of the limbs was immobilized, and the rabbits were allowed to exercise ad libitum in cages. Eight rabbits in each group were sacrificed by injecting thiamylal sodium under pentobarbital anesthesia at 3 and 6 weeks after surgery. In the stress-shielded group, it was confirmed at sacrifice that the patellar tendon was relaxed at all knee angles. Six and two of eight rabbits in each group were used for ultrastrutural and light microscopic evaluations, respectively.

Surgical procedures

Surgery was performed under intravenous pentobarbital anesthesia (0.05 mg/kg). After the anterior region of the right knee was exposed through a midline longitudinal skin incision, the medial and lateral retinacula were incised longitudinally along both patellar tendon edges. Tension in the patellar tendon in the stress-shielded group was completely released in the same fashion as that reported previously.[10,11,17,22] Briefly, each animal was fixed to a specially designed table, with the right knee flexed to 90°. Two stainless steel pin markers (0.7 mm diameter) were embedded in the patella and the tibial tubercle, and the distance between them was measured by a caliper. In addition, a stainless steel pin (1 mm diameter) and a stainless steel screw (3 mm diameter) were inserted into the patella and the tibial tubercle, respectively. A stainless steel wire (0.97 mm diameter) was installed between the pin and the screw. The patellar tendon was slackened by pulling the stainless steel wire. When the distance between the two markers was shortened by more than 6 mm, both edges of the wire were firmly fixed. In previous studies[10,11,17,22] we confirmed that the patellar tendon was relaxed at all knee flexion angles and that no stress was applied to the patellar tendon throughout the experimental period. The same surgical procedure was performed on the animals of the sham group. However, the wire installed between the patella and the tibial tubercle was not tightened so the patellar tendon was not shielded from stress.

Microstructural and ultrastructural observations

Patella–patellar tendon–tibia complexes were taken out of the knees immediately after sacrifice, and their surrounding tissues were carefully removed. For light microscopy (XF-15; Nikon, Tokyo, Japan) evaluation, these complexes were then fixed in a buffered 10% formalin solution, decalcified, and cast in paraffin blocks. The specimen blocks including osseous attachments were sectioned along the longitudinal axis of the patellar tendon. The sectioned specimens were stained with hematoxylin and eosin for light microscopy.

Ultrastructural observation and analysis was performed in the same fashion as that reported previously.[14,19] Briefly, the central one-third of each patellar tendon was divided into six subblocks: dorsoproximal, dorsomiddle, dorsodistal, ventroproximal, ventromiddle, and ventrodistal. A 1 mm thick specimen sliced perpendicular to the patellar tendon axis was cut from each subblock, fixed in a 2.5% buffered glutaraldehyde solution, and postfixed in 1% osmium tetroxide buffer solution. The specimen was dehydrated in graded alcohols and embedded in epoxy resin. Thick sections approximately 0.5 μm were cut from each specimen perpendicular to the long axis; ultrathin sections about 80 nm thick were similarly cut. The sections were stained with uranyl acetate and lead citrate for transmission electron microscopy (TEM) (JEM-100CX; Nihon Denshi, Tokyo, Japan).

Using a light microscope, we carefully observed the entire cross section (coronal plane) of each subblock obtained from the central one-third of the patellar tendon (magnification 100×). The image was captured with a video camera followed by a video analyzer (Percept Scope-C3160; Hamamatsu Photonics, Tokyo, Japan) to measure the number of cells observed in a visual area of 1 mm^2.

For quantitative collagen fibril analyses, a randomly selected electron micrograph was taken at a final magnification of 41 000× from each of six subblocks obtained from each patellar tendon. The diameters of all collagen fibrils in a 9 μm^2 area randomly chosen from each micrograph were measured with image analysis software (Cosmozone-1S; Nikon, Tokyo, Japan). A histogram of the collagen fibril diameters for each patellar tendon was obtained from a summation of the results of the analyses for the six subblocks. For each group, an average histogram was also made at each period by calculating the histogram data obtained from six patellar tendons. The number of collagen fibrils in a 1 μm^2 area was also determined. In addition, the ratio of the total cross-sectional area of collagen fibrils to the whole visualized area was defined as the fibril occupation ratio, and this value was calculated for each patellar tendon.

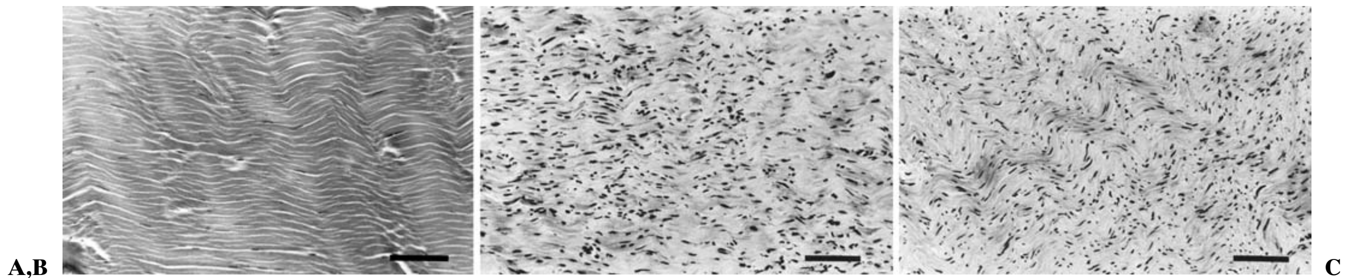

Fig. 1. Micrographs of the longitudinal sections on the sagittal plane of the control and stress-shielded patellar tendons. The upper and lower sides of each micrograph correspond to the ventral and dorsal sides, respectively. **A** Central portion of the patellar tendon in the control knee. **B** Central portion of the patellar tendon in the 3-week stress-shielded group. **C** Central portion of the patellar tendon in the 6-week stress-shielded group. Note a number of proliferated round and ovoid cells without consistent orientation and the disorganized and loose collagen. *Bars* 100 μm

Table 1. Cell nuclei in patellar tendons

Group	Cell nuclei/mm²		
	3 Weeks	6 Weeks	Total
Control			439 ± 55
Sham	410 ± 67	459 ± 92	
Stress-shielded	2383 ± 198*,**	2053 ± 148*,**	

Results (mean ± SD) and the averaged number of cell nuclei/mm² in five areas in control, sham, and stress-shielded patellar tendons
*Significant versus control ($P < 0.05$); **significant versus sham ($P < 0.05$)

Statistical analysis

We used two-way analysis of variance (ANOVA) to compare the effects of time (3 and 6 weeks) and treatments on the median of the diameter, the number of collagen fibrils in the unit area of 1 μm², and the fibril occupation ratio. When significant effects were confirmed by ANOVA, all possible post hoc comparisons were made using Tukey's method. The significance level was set at 0.05.

Results

Microstructural observations

The histology of the control patellar tendon was characterized by longitudinally oriented collagen fibers with a periodic crimp pattern and spindle-shaped fibroblasts scattered in the matrix (Fig. 1A). There were no histological differences between the sham and control groups throughout the experimental period. In the 3-week stress-shielded tendon, however, we observed cells with ovoid or plump nuclei, and collagen bundles were less well oriented than in the sham and control groups (Fig. 1B). At 6 weeks, many ovoid cell nuclei were still observed in the stress-shielded group. Collagen bundles in the stress-shielded tendon were coarser and less well oriented than those in the sham group (Fig. 1C). The crimp pattern of collagen bundles was less well oriented than in the control tendon. More leukocytes, macrophages, and capillary endothelial cells were observed in the stress-shielded patellar tendon than in the control tendon.

The average values in five areas in each group at each period of the number of cells observed in a visual area of 1 mm² are shown in Table 1. The number of cells in the sham-operated tendon at each period was almost equal to that seen in the control tendon. In the stress-shielded tendon, the number of cells increased markedly to about five times that in the control tendon at 3 weeks, although the number did not change thereafter.

Ultrastructural observations

In the control tendon, the histogram of collagen fibril diameters was bimodal; it ranged from 30 to 330 nm, with two peaks at 60 and 180 nm (Figs. 2A, 3A). The median was 161.4 nm. The number of collagen fibrils in the 1 μm² area averaged 24.4, and the fibril occupation ratio averaged 68.0% (Table 2). The histogram and all measured parameters of the sham group were similar to those in the control tendons. There were no significant differences in the quantitative parameters from ultrastructural observations between the control and

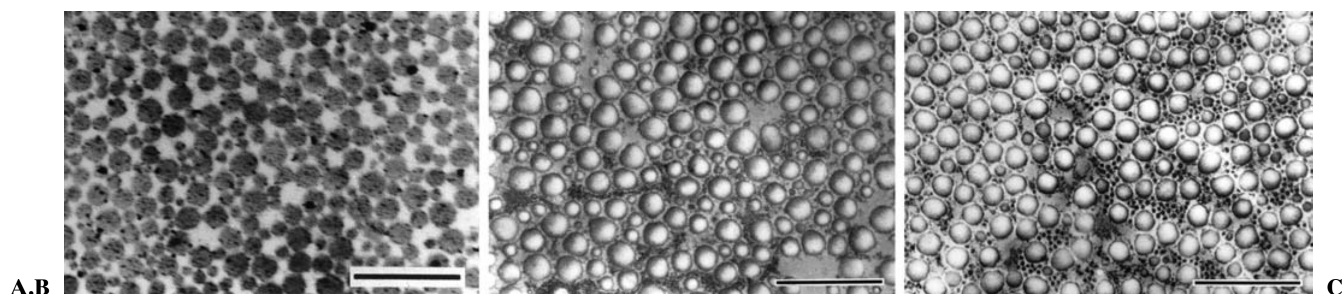

Fig. 2. Electron micrographs of the patellar tendon. **A** Normal patellar tendon. **B** Three-week stress-shielded patellar tendon. **C** Six-week stress-shielded patellar tendon. Note that small collagen fibrils are predominant. ×10 000, *Bars* 1 μm

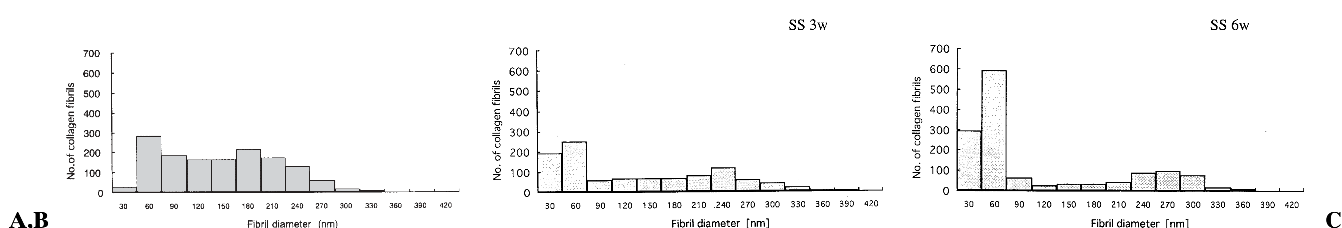

Fig. 3. Histograms of the diameter of collagen fibrils. **A** Normal patellar tendon. **B** Three-week stress-shielded patellar tendon. **C** Six-week stress-shielded patellar tendon

Table 2. Quantitative ultrastructural observations

Parameter	3 Weeks	6 Weeks	Total
Median of fibril diameter (nm)			
Control			161.4 ± 5.1
Sham	158.2 ± 14.6	156.7 ± 10.6	
Stress shielded	146.3 ± 28.7	94.4 ± 19.7*,**,***	
No. of fibrils/μm²			
Control			24.4 ± 1.4
Sham	24.6 ± 3.1	24.2 ± 2.1	
Stress shielded	17.6 ± 2.1*,**	26.5 ± 3.5***	
Fibril occupation ratio (%)			
Control			68.0 ± 1.1
Sham	67.7 ± 0.9	66.5 ± 1.9	
Stress shielded	49.0 ± 3.2*,**,***	55.1 ± 0.9*,**,***	

Results are the mean ± SD
* Significant versus control ($P < 0.05$); ** significant versus sham ($P < 0.05$), *** significant versus 3 weeks ($P < 0.05$)

sham groups (Table 2). At 3 weeks, there was no significant difference in the median of the collagen fibril diameters between stress-shielded tendon and the sham-operated tendon. However, the histogram of the 3-week stress-shielded tendon showed an increase in the larger-diameter fibrils (the maximum value was 240 nm) when compared to the sham and control tendons. The number of collagen fibrils in a 1 μm² area decreased 3 weeks after the stress-shielding treatment. The fibril occupation ratio was significantly smaller in the 3-week stress-shielded tendon than in the control and sham tendons (Figs. 2B, 3B). At 6 weeks, small collagen fibrils with a diameter less than 90 nm were predominant in the stress-shielded tendon. The histogram of the 6-week stress-shielded tendon ranged from 30 to 360 nm, with two peaks at 60 and 270 nm. The median of the collagen fibril diameter in the 6-week stress-shielded tendon was significantly smaller than in the control tendon (58.8% of control), resulting in a significant decrease in the fibril occupation ratio. In contrast, there was no significant difference between the control and stress-shielded groups at 6 weeks regarding the number of collagen fibrils in a unit area of 1 μm² (Figs. 2C, 3C; Table 2).

Discussion

In the present study, it was shown that there were significant differences in the number of cells and the diameter of collagen fibrils between the sham-operated patellar tendon and the completely stress-shielded patellar tendon. Thin collagen fibrils with a diameter of 90 nm or less were predominant in the stress-shielded patellar tendon. The median of the collagen fibril diameters determined in the 6-week stress-shielded patellar tendon was significantly smaller than that measured in the sham-operated tendon (58.8% of control).

This study demonstrated that complete stress deprivation significantly increases the number of cells in the unit area. The cells were varied and included leukocytes, macrophages, fibroblasts, and capillary endothelial cells. These cells may affect the collagen fibrils of the patellar tendon. Inflammatory cells involving macrophages may produce collagenase, resulting in a decreased collagen fibril diameter. Infiltrating immature fibroblasts may produce small-diameter collagen fibrils. Furthermore, stress shielding may directly affect the collagen fibril diameter.

Binkley and Peat[4] observed ultrastructural changes in the rat medial collateral ligament caused by immobilization. They have reported that thin fibrils were decreased and thick fibrils were increased by immobilization. They speculated that reduced stress decreased collagen synthesis, resulting in delayed turnover of collagen fibrils. In the present study, stress shielding changed the higher peak of the fibril diameter in the histogram. This result is consistent with that of a previous report.[4] In contrast, the present study showed that small collagen fibrils with a diameter of less than 90 nm were predominant in the stress-shielded patellar tendon. The median collagen fibril diameter in the 6-week stress-shielded patellar tendon was significantly smaller than that in the control tendon (58.8% of control). These results may be attributable to differences in immobilization and stress deprivation. That is, immobilization causes not only stress deprivation but also loss of joint motion, and the stress-shield treatment applied in the present study can remove stress from the tendon, allowing joint motion. Another possible reason for the differences between the present study and the previous one[4] is the difference between the tendon and ligament. That there were no significant differences in the quantitative parameters from the microstructural and ultrastructural observations between the control and sham groups at 3 and 6 weeks indicate that the effect of surgical treatment for stress shielding can be ignored.

In the previous study using the same model,[11,22,23] we reported that complete stress deprivation significantly reduced the tensile strength and tangent modulus of the patellar tendon. Moreover, the deterioration of these mechanical parameters depended on the degree of stress reduction. Doillon et al. reported that there is a direct relation between the collagen fiber diameter and the tensile strength in dermal repair tissues.[6] The present study provided evidence that the changes in the collagen diameter observed in this study may explain the reduction in the mechanical properties reported in the previous study with the same experimental model.

In previous studies, we reported that stress shielding significantly increases the collagenase mRNA level of the medial collateral ligament using an organ culture model.[12,13] Stress deprivation increases collagen turnover of the ligament matrix,[2,7] and stress shielding increases the degrading enzymes of collagen fibers.[7,18] It has also been reported that collagenase reduces the collagen fibril diameter in the rabbit medial collateral ligament.[5] These studies indicate that stress deprivation may enhance the catabolism of the tendon, which in turn may explain the results of the present study.

Conclusions

This study showed that complete stress deprivation on the patellar tendon increased the small-diameter collagen fibrils in the rabbit model. Further biological experiments should be conducted to understand this mechanism.

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