Appropriate Tensile Mode and Timing of Applying Tension to Promote Tendon Gel Regeneration

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Abstract “Tendon gel” secreted from a parent tendon is regenerated for tendon repair by applying tension. However, the details of the tensile stimulus have not been clarified. This study aimed to evaluate an appropriate tensile stimulus mode and the optimal timing of applying tension to promote tendon gel regeneration. Tendon gel was prepared using a film model method in mice and was preserved *in vivo* for 3, 5, and 10 days. Unlike tendon gel on day 3 or day 5, a fibrous structure developed in the tendon gel on day 10 when tension was applied. Infrared spectroscopy revealed that characteristic peaks appearing for the tendon gel on days 3 and 5 disappeared on day 10. Disappearance of the peaks indicated maturity of the tendon gel, and it showed the optimal timing for tension application to the tendon gel. The effect of tensile load on tendon gel preserved for 10 days was investigated using a tensile test, a creep test, or a cycle test. In the tensile test, tendon gel was elongated into a thin cord of collagen fibers with an increase in stress, and the maximum diameter of the collagen fiber was approximately 50 times larger than that in the normal Achilles tendon of mice. The results suggest that the diameter of the oriented collagen fiber is controllable by adjusting the applied load and the time in mature tendon gel.

Keywords Collagen fiber · Tensile stress · Structural control · Atomic force microscope · Fourier transform infrared

1 Introduction

Collagen is a major protein found in the dermis, tendon, ligament, bone, and cartilage. In the normal tendon, the collagen fibril synthesized by tendon fibroblasts forms a unidirectional, aligned structure, and then forms tough

cords of collagen fibers that connect bone and skeletal muscle fibers [1, 2]. The tensile strength of the human Achilles tendon has been estimated to be 50–100 N/mm² [3, 4]. An excessive load of mechanical stress on the tendon causes tendon injuries. Because more middle aged and older people are participating in various sports, it is supposed that the number of patients with tendon or ligament injuries will increase year by year. For tendon injuries, repair by surgery or conservative treatment is selected because tendons have the ability to self-heal, whereas rebuilding with a transplant is usually conducted for anterior cruciate ligament damages [5–7]. Therefore, much attention has been paid to the anatomical or morphological analysis of repairing the injured tendon [8–12], and the development of a repair technique based on stem cells or collagen fiber scaffolds has been reported [13, 14].

Recently, we have investigated the regenerative process of the Achilles tendon in mice using a film model method [15], and we found that a translucent gel-like substance,

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which we called “tendon gel”, was secreted from the parent tendon and acted as the key material of tendon repair [15]. The tendon gel was originally an amorphous-like structure where collagen fibril was undeveloped, and regeneration of the tendon gel was begun on the specimen preserved more than 10 days *in vivo* by applying tension. Aligned collagen fibrils emerged along the tensile direction in the gel region [15].

The importance of mechanical force for the repair of injured tendons has already been reported [16, 17]. However, regeneration conditions such as appropriate tension and its timing to promote regeneration of tendon gel have not been clarified yet. We will approach these issues using nondestructive structural analysis techniques. In recent years, in research on biomaterials or regeneration, the use of infrared spectroscopy [18, 19] and a scanning probe microscopy [20–25] have been reported, and they have been made available. Fourier transform infrared (FT-IR) spectroscopy will provide information on the change in collagen molecular structure [18, 19]. On the other hand, atomic force microscopy (AFM) will provide information on the formation of collagen fibrils [20–25]. The employment of these pieces of equipment will enable the evaluation of the optimal timing of the formation of collagen fibrils using a structural analysis of tendon gel.

In general, there are three ways to test the tensile mode of the materials: a monotonous increase load [26, 27], a static load of constant loading [28], and a repeat of the constant load [29–31]. The evaluation of the effect of these tensile modes on regeneration of tendon gel might lead to the adoption of the optimal tensile mode.

We believe that an artificial tendon could be formed by applying the optimal tension with the appropriate timing to tendon gel. In this study, the following questions were investigated: (1) Why is tendon gel preserved *in vivo* for more than 10 days regenerated by applying tension? (2) What is the optimal method to apply tension to promote the regeneration of tendon gel? Investigations on the appropriate timing and tensile mode of applying tension to tendon gel will enable controlling the regeneration structure of tendon gel, including the thickness and orientation of the collagen fibers. Analysis of the regeneration mechanism of tendon gel will provide useful information for solving the clinical challenge of tendon repair and the formation of artificial tendon. The hypotheses in this study were as follows. First, the structural characterization of tendon gel using FT-IR spectroscopy and AFM on each gel specimen preserved *in vivo* for 3, 5, and 10 days before and after applying the tension indicates the optimal timing at which tension should be applied to promote regeneration of the tendon. Second, measuring the diameter of collagen fiber in

tendon gel during each tensile testing mode indicates the appropriate tensile mode.

2 Materials and methods

2.1 Experimental strategy

In this study, the effect of the tensile stimulus on tendon gel was evaluated using the following experimental strategies. First, we employed a film model method to prepare the specimens. The film model method enabled structural evaluation by formation of a thin specimen in the gap between fluorine films, without making a graft specimen. The second was employment of the originally fabricated testing system for applying tension to the tendon gel. The third method we used to evaluate the effect of the tensile stimulus was FT-IR and AFM for the structural characterization. The experimental strategies are summarized in Fig. 1. In a conventional optical microscope observation, the resolution is inadequate for observation of the collagen fibril development [32]. In the case of a transmission electron microscope observation, high technical standards are required for specimen preparation. We used FT-IR measurements and AFM observations to provide information on the molecular level tendon gel structure without damage to the biostructure and a special technique for specimen preparation.

2.2 Specimen preparation

Animal care and procedures were approved and performed in accordance with the standards set forth by the University Animal Care and Use Committee (Approval number: 143031, 154004). In this experiment, 27 adult male ddY mice (young adult mice, 6 weeks old, 25–30 g body weight, Japan SLC, Shizuoka, Japan) were used for the sample.

Specimen preparation was conducted by an expert who specializes in anatomy, and has 8 years of extensive experience in the preparation of tendon gel specimens. The tendon gel specimen was prepared based on a film model

Fig. 1 Schematic illustration of experimental strategies

method [15, 33]. This method involved sandwiching the transected tendon between two pieces of a fluorine resin film of approximately 4.2×3.5 mm (Aflex, Asahi Glass Co., Ltd., Tokyo, Japan). The Achilles tendon was transected with fine scissors at the middle point of the insertion (tendon) connecting to the medial head of the gastrocnemius [15]. The proximal end of only the collagen bundle that eliminated a part of the tendon sheath after the transected tendon was placed on a film sheet and anchored with a single 10-0 nylon suture (Matsuda Sutures, Tokyo, Japan). Only the proximal end of the collagen bundle was placed on the film after tenotomy. The operated tendon was then moistened with several drops of Ringer's solution and covered with another sheet of film. The two films were sewn together so that the collagen bundle was sandwiched between them. At 3, 5, and 10 days after the film model procedure, mice were sacrificed using a lethal dose of avertin. The number of mice used for the day interval groups of 3, 5, and 10 days was 4, 5, and 18, respectively. Based on our previous report [15], the day-interval groups were decided based on specimen size and the maturity of tendon gel. Because the orientation of the collagen fibers occurred in tendon gel preserved *in vivo* for more than 10 days [15], tendon gel preserved *in vivo* for 10 days was estimated as the threshold. A considerable amount of 10-day tendon gel was used for the experiments. The experiment was conducted in more than two tendon gel specimens to confirm reproducibility of the structure. Before applying tension, AFM observations and IR measurements were performed with two specimens for each day-interval group, and after applying the tensile stress of approximately 0.5 MPa, we used 2, 3, and 3 specimens for the 3-, 5-, or 10-day intervals, respectively (a total of 14 specimens). The number of tendon gel specimens for the tensile test was 7 pieces. Three tendon gel specimens were used for the creep test and 3 for the cycle test. After preservation for 3, 5, and 10 days *in vivo*, a translucent gel-like substance was formed as the tendon gel, we then excised the gel specimen as shown in Fig. 2. After

removing the films, only the tendon gel was used as a specimen for structural characterization or tensile tests. Tendon gel preserved *in vivo* for 2 days was determined to be too small for experimental use.

2.3 Structural analysis

Surface observation of the specimens was conducted using AFM (Dimension 3100, Veeco, USA). In the AFM observation, the surface structure was difficult to observe due to the distribution of migrating tendon gel productive cells (fibroblasts). For the removal of the fibroblasts distributed on the gel specimen, we flushed out migratory cells from the parent tendon by applying ethanol to the specimen. An NCHV-10 V cantilever was used for surface observation with the tapping mode. Structural analysis was carried out using microscopic FT-IR (IRT5000, FT/IR4200A, JASCO, Japan). In the transmission measurement, the aperture diameter was 50×50 μm and the integration count was 40 times. AFM observations and IR measurements were conducted on tendon gel preserved *in vivo* for 3, 5, or 10 days before and after applying a tension of approximately 0.5 MPa, as well as on the normal Achilles tendons of mice. The specimens for AFM observations or FT-IR measurements were placed on the glass substrate with moisture. Since tendon gel is sensitive to mechanical stress, the tendon gel structure may be affected by handling during specimen preparation. In particular, at the specimen installation of the tensile tests, the fibrous structure of collagen is possibly formed near the parts that are tied with a nylon suture or the parts that are mounted by pricking them on a hook pin. In consideration of this likelihood, structural characterization was conducted that excluded these specific regions.

2.4 Test for applying the tension

Evaluation of the relationship between various tensile modes and the obtained structures will lead to

Fig. 2 Schematic illustration of a film model method and photograph of a typical tendon gel specimen

determination of an appropriate tensile mode for regeneration of tendon gel. In this study, we used a conventional precise tensile test (tensile testing) that applied an increased monotonic load, a static tension test (creep testing) that applied a constant load continuously, and a cyclic tension test (cycle testing) that applied a constant load repeatedly. These tests were conducted using originally designed testers, as shown in Fig. 3A–C. Each tester was combined with an optical microscope attached to a charge coupled device camera (Moticam 2300, Shimadzu Corp., Kyoto, Japan) and a load-measuring device. The dimensions of the tendon gel specimen were different when stratified by the preservation days *in vivo* and by millimeter order. The thickness of tendon gel was measured by using a micrometer (ID-C112B, Mitsutoyo, Japan).

The tensile test was conducted on tendon gel preserved for 3, 5, and 10 days *in vivo*. In the tensile test, a tensile unit that consisted of a fixed stage and an originally designed movable stage with a load detection sensor was installed in a specimen holder as shown in Fig. 3A [34]. Between the stages, the tendon gel specimen was mounted on the tensile unit by pricking the specimen on a hook pin. The initial distance between the hook pins was approximately 0.5 mm. The maximum measurement load of the unit was approximately 2 N and the load resolution of the sensor was 10^{-5} N. The tensile testing speed was approximately 20 $\mu\text{m/s}$.

The creep test or the cycle test was conducted on tendon gel preserved for 10 days *in vivo*. The number of tendon

gel specimens for each test was 3 pieces, respectively (total 6 specimens). The creep testing unit with a digital force gauge (FGP-0.5, NIDEC-SHIMPO Corp., Kyoto, Japan) and the cycle testing unit with an actuator (TD-102, Techonohands Co., Ltd., Kanagawa, Japan) were originally assembled for each tester, as shown in Fig. 3B, C. In the creep test and the cycle test, the ends of the tendon gel specimen were tied with a single or loop-shaped 10-0 nylon suture for applying the stress. Then the specimen was dipped in a petri dish that was filled with Ringer's solution. After loosening the nylon suture before the tests, we set the zero position. Stress of approximately 0.5 MPa for the creep test was applied, controlling the specimen displacement at approximately 0.5 mm. We applied stress of approximately 0.5 MPa for the cycle test using steps of 360 times/h with displacement of 0–0.5 mm. The stress-loading pattern in both the creep test and the cycle test is shown in Fig. 3D.

3 Results

3.1 Tendon gel prepared by the film model method

Figure 4 show optical micrographs of the tendon gel specimens taken from mice. By sandwiching the proximal end of the Achilles tendon that removed the tendon sheath with the fluorine films, the amount of tendon gel increased and spread into the gap of the films with the passing of 3, 5,

Fig. 3 Photographs and schematic illustrations of tensile testers: a precise tensile test **A**, static tension applying test (creep test) **B**, and a cyclic tension applying test (cycle testing) **C**. The tensile stress pattern of the creep test and cycle test **D**

Fig. 4 Optical micrographs of tendon gel specimens taken from mice. The tendon gel specimen matures *in vivo* on day 3 **A**, day 5 **B**, and day 10 **C**

or 10 days. The average dimensions of the tendon gel specimens on days 5 and 10 were approximately 3×2 mm, and that of the specimens on day 3 was slightly smaller: approximately 2×2 mm. The approximate average thickness of the tendon gel specimens on days 3, 5, and 10 was $35.8 \pm 8.2 \mu\text{m}$ ($n = 20$), $16.0 \pm 4.2 \mu\text{m}$ ($n = 20$), and $22.8 \pm 1.1 \mu\text{m}$ ($n = 20$), respectively. The tendon gel on day 5 spread more widely, with high density, compared with the gel on day 3. On the other hand, the gel specimens on day 5 and on day 10 had almost the same structure at the optical microscopic level, as shown in Fig. 4B, C. Structural observation using AFM was conducted at the molecular level to clarify the relationship between preservation days and tendon gel structure. After removing the migratory cells on the tendon gel, we clearly observed the tendon gel's surface structure.

3.2 Structural characterization of the tendon gel specimen on each day interval groups

Every tendon gel sample preserved *in vivo* for 3, 5, or 10 days had a homogeneous, amorphous-like structure where collagen fibrils were undeveloped and a fibrous structure was not observed. A typical tendon gel structure on day 10 is shown in Fig. 5A. In this experiment, the tensile tester put stress of approximately 0.5 MPa on the tendon gel on day 3 or day 5 for 1 h. However, the samples did not become fibrous. Figure 5B shows a typical tendon gel on day 5 after applying the tension.

On the other hand, tendon gel on day 10 showed that fibrous structure with striation, which was a peculiar feature of collagen fibrils, was developed in the same tensile conditions, as shown in Fig. 5C. The mean diameter of collagen fibrils in the observed area was approximately 54 ± 20 nm. The observed collagen fibrils were also aligned in the tensile direction. The experimental results were consistent with those of our previous report [15].

Figure 6A–G show a series of FT-IR spectra of tendon gel specimens before and after applying the tension, and a

normal Achilles tendon of a mouse. The right-hand side of the diagram (around the $1500\text{--}1000\text{ cm}^{-1}$ region) contains a complicated series of absorptions that is called the fingerprint (Fig. 6A, B). This is mainly due to a variety of bending vibrations within the molecule. However, we found that the fingerprint region of the tendon gel on day 10 was different from that of the specimen on day 3 or day 5. That is, a peculiar peak in the fingerprint region of the tendon gel on day 10 disappeared as shown in Fig. 6A–C. This result indicates that the molecular structure of tendon gel preserved *in vivo* for 10 days is different from that of preserved for 3 and 5 days. The IR spectrum change suggested that the tensile stress should be applied to the tendon gel specimen preserved for 10 days to obtain the optimal timing.

Figure 6C–E show FT-IR spectra of tendon gel specimens before and after applying the tension, and the normal Achilles tendon of a mouse measured in this experiment. After applying the tension to the tendon gel on day 10, a peak indicating the presence of the $-\text{CHO}$ group was confirmed near 1750 cm^{-1} at an arrowed peak (Fig. 6D, F). On the other hand, the peak indicating the $-\text{CHO}$ group was not detected from a normal Achilles tendon of a mouse (Fig. 6E, F). Furthermore, the IR spectrum showed that the peak position of the $-\text{OH}$ group shifted to the high-wave-number side with the normal Achilles tendon as shown in an arrowed peak in Fig. 5G.

The clear spectrum change of the gel specimen on day 3 or day 5 was not confirmed before and after applying the tension.

3.3 Regeneration of tendon gel by tensile test

In the tensile test that increasingly applied a monotonic load, both ends of the gel specimen were pricked on a hook pin and fixed on the tensile testing unit. As control experiments, tensile tests were performed in tendon gel preserved for 3 or 5 days. The specimen on day 3 was ruptured near the hook pin without elongation by applying

Fig. 5 Atomic force microscope images of tendon gel. Typical tendon gel structure on day 10 **A**. A typical tendon gel on day 5 after applying the tension **B**. In a specimen that was preserved *in vivo* for 10 days after applying a tensile stress of approximately 0.5 MPa, the fibrous structure with the striation that is a peculiar feature to collagen fibrils is exhibited **C**

Fig. 6 FT-IR spectra of tendon gel specimens before and after applying the tension, and the Achilles tendon of a normal mouse. The right-hand side of the diagram (around the 1500 to 1000 cm^{-1} region) contains a complicated series of absorptions called the fingerprint **A**, **B**. However, the fingerprint region of the specimen on day 10 disappears, unlike the specimen on day 3 or day 5 **C**. Collagen molecules begin cross-linking by applying tension to a gel specimen

kept *in vivo* more than 10 days **D**. The cross-linking reaction is known as an aldol condensation cross-linking reaction **E**. The presence of $-\text{CHO}$ appears near 1750 cm^{-1} at the arrowed peak **F**. Since the $-\text{CHO}$ group is consumed in the Schiff base cross-linking reaction, the peak originating from $-\text{CHO}$ group disappears when the tendon is finally reproduced **F**, and the peak position of the $-\text{OH}$ group shifts to the high-wave-number side at the arrowed peak **G**

the tensile stress, and the specimen on day 5 was ruptured with little elongation, as shown in Fig. 7A–D. However, the specimen on day 10 was elongated with an increase of the tensile stress, as shown in Fig. 7E–G. Only mature tendon gel specimens that were preserved *in vivo* for 10 days were elongated into a string-like shape from the sheet-shaped tendon gel without rupturing in this experimental condition.

The load displacement (LD) curve of the tendon gel on day 10 is shown in Fig. 7H. The tensile load increased with an increase of displacement (plastic deformation) of the tendon gel specimen. Since increased loads tend to increase strain, the observed mechanical behavior was similar to typical work hardening observed in common metal materials. The behavior of the LD curve exhibited a sawtooth-like fluctuation with an increased load.

Fig. 7 A series of conventional tensile tests of tendon gel specimens on day 3, 5, and 10. The specimen on day 3 is ruptured near the hook pin without elongation from the tensile stress **A, B**, and the specimen on day 5 is ruptured with little elongation **C, D**. The specimen on day 10 becomes a string-like shape with an increase of the tensile stress **E–G**. The load–displacement curve of the specimen on day 10

exhibits sawtooth-like fluctuation with an increase of the load **H**. The diameter of the observed collagen fiber [black arrows] is approximately 10 μm , and the thickness is approximately 40 times that of normal mice Achilles tendon **I**. The black arrows with white bordering on the figures show the tensile direction

Figure 7I shows an optical microscope image of the string-like specimen shape produced by the tensile test on day 10. The length of the tendon gel varied from approximately 0.5 to and 3.3 mm with tensile stress of approximately 9 MPa, and the thinnest diameter of the elongated tendon gel (a string-like shape) was approximately 135 μm .

When the maximum tensile stress (approximately 9 MPa) was applied, the diameter of the observed collagen fiber in the elongated tendon gel was approximately 10 μm , and the diameter of the fiber was approximately 50 times thicker than that of the observed normal Achilles tendon of mice with an average diameter of approximately $189.4 \pm 70.2 \text{ nm}$ ($n = 11$). The diameters of the collagen fibers were not necessarily constant depending on species, age, and sample location. These data were the average diameter measured from the Achilles tendon of mice that excised tendon gel. The relationship between the diameter of collagen fibrils and the applied stress in the tensile tests

is shown in Fig. 8. The diameter of the collagen fiber instantly and exponentially became thicker with an increase of stress. This means that the increase of stress on the gel enhances promotion of the cross-linking reaction between collagen molecules. In the tensile test, however, it should be noted that a large morphological change of tendon gel occurs.

3.4 Regeneration of tendon gel by creep test and cycle test

Figure 9A, B show the optical micrograph of the gel specimen before and after the creep test that applied a constant load continuously. The whole specimen was extended by approximately 25%, but the original shape of the specimen was almost kept. AFM observations after the test showed that collagen fibers were aligned in the direction that applied the stress (Fig. 9C). The mean diameter of the collagen fibers was approximately $208.3 \pm 51.3 \text{ nm}$

($n = 28$). However, after the cycle test, the mean diameter of the developed collagen fibers was almost half [approximately 98.6 ± 44.8 nm ($n = 40$)] as shown in Fig. 9D–F. The diameter of collagen fiber obtained using the creep tests was approximately two times than that of the cycle test ($p < 0.01$, one-way analysis of variance). The mean diameters of the collagen fibers in tendon gel are summarized in Table 1. Because the diameter of the collagen fibers, which was equivalent to the diameter of mice collagen fiber, was formed without a significant morphological change, the application of the creep test was effective for the regeneration of tendon gel.

Fig. 8 Relationship between the diameter of collagen fibers and applied stress in the tensile tests

4 Discussion

We conducted an evaluation of the optimal timing to promote regeneration of tendon gel by structural characterization using FT-IR spectroscopy and AFM, and the investigation of an appropriate tensile mode by originally fabricated tensile testers. Our experimental results showed that tendon gel preserved for 10 days *in vivo* was regenerated by applying tension, whereas tendon gel preserved for 3 or 5 days *in vivo* did not form collagen fibrils, even when applying tension. The formation and orientation of the collagen fibrils when the tension was applied depended on whether collagen molecules matured in the gel preserved. Investigation of the maturity of tendon gel using spectroscopy enabled an evaluation of the optimal timing that tensile stimulus should be applied to the gel.

FT-IR spectrum analysis showed that disappearance of a peculiar peak in the fingerprint region. The difference, shown in Fig. 6A–C, may be due to a cleavage of the propeptide of procollagen, synthesized by tendon cells [35]. Disappearance of the fingerprint indicated maturity of the tendon gel, which tells us the optimal timing that the tensile stress should be applied to the tendon gel.

These results demonstrate that FT-IR is an effective tool for confirmation of the maturity of tendon gel. The fibrosis structure in tendon gel is caused by cross-linkage between

Fig. 9 Optical micrographs of the tendon gel before and after a creep test or a cycle test and AFM images after the tests. In the creep test, the whole specimen was also extended approximately 25% **A, B**. An AFM image after the creep test showed that collagen fibers were aligned in the direction that applied the stress **C**. In the cycle test, the

whole specimen was extended by approximately 20% **D, E**. An AFM image after the test showed that collagen fibers were also aligned in the direction that applied the stress **F**. The growth of collagen fibers by the creep test was more advanced than that by the cycle test. The white arrows on the figures show the tensile direction

Table 1 The diameter of each collagen fiber in this experiment

Tensile modes	Average diameter (nm)	SD (nm)	Number of collagen fiber (n)
Cycle test	98.6	44.8	40
Creep test	208.3	51.3	28
cf.) Normal Achilles tendon of mice	189.4	70.2	11

collagen molecules, and the chemical substances produced during each cross-linking reaction have also been identified [36–38]. Due to the detailed understanding of the regenerative process of the tendon, it is necessary to characterize the initial process of the structural change in tendon gel specimens and the normal tendon of mice.

Based on the cross-linking models [36–38], the FT-IR spectrum is explained as follows: the first cross-linking reaction is known as an aldol condensation cross-linking reaction. In this reaction, a saturated fatty acid aldehyde group (–CHO group) was formed [36], and a peak indicating the presence of the –CHO group was confirmed near 1750 cm^{-1} at an arrowed peak (Fig. 6D, F).

The cross-linking of collagen molecules began when we applied tension only to the tendon gel specimen that was preserved *in vivo* more than 10 days. The onset of the peak near 1750 cm^{-1} corresponds with formation of the oriented collagen fibrils by the cross-linking of collagen molecules. Then, when the cross-linking reaction advanced, collagen bundles were formed by a Schiff base cross-linking reaction, and the bundles became thicker with the passing of the days. In the Schiff base reaction, the structural model shows that the molecular structure of the –OH group neighborhood changed (site shift), as shown in Fig. 9B, and this change may contribute to a shift of peak position originating from the –OH group in the IR spectrum. Since the –CHO group was consumed for the Schiff base cross-linking reaction [37–39], the peak originating from the –CHO group disappeared when the tendon was finally repaired (Fig. 6E, F). It became clear that the peak position of the –OH group shifted to the high-wave-number side at an arrowed peak (Fig. 6G). These results show that the regeneration process of tendon gel such as the onset of the cross-linking reaction of collagen molecules and the initial stage of the regeneration process of the tendon gel could be confirmed by FT-IR measurements and AFM observations.

As mentioned above, FT-IR and AFM results showed us the optimal timing that tension should be applied to the tendon gel. Then, an appropriate mode to apply the tension was investigated using various tensile modes on tendon gel preserved *in vivo* for 10 days.

In the tensile test, the fluctuation of the LD curve shown in Fig. 7H may be due to local rupture during

elongation of the gel specimen. The load increase during the tensile tests (work hardening) might be caused by tensile stress-induced transformation of the gel specimen. The mechanical strength of the gel specimen will be increased by promotion of the cross-linking reaction between the collagen molecules and subsequent structural changes such as orientation of the collagen fibrils in the tensile direction and growth of collagen bundles.

The diameter of collagen fiber obtained by the creep test was almost the same as that of the normal Achilles tendon of mice. However, the diameter of the collagen fiber obtained by the cycle test was about half of the normal Achilles tendon of mice as shown in Table 1. The difference of the diameter of collagen fiber may be due to a difference of the total time when tension was applied. In the cycle test, the total time when tension was applied was approximately 70% due to an interval of applying the tension compared with the creep test. The result suggests that the cross-linking of collagen molecules developed only when the tensile stress was applied. In repair of tendon injuries, it has been reported that mechanical loading would enhance tendon repair and remodeling by stimulating tendon fibroblast activities [40]. Our experimental result indicated that applying tension promotes cross-linking of collagen molecules in the tendon gel. As the results of these experiments show, applying a constant load (the creep tensile mode) on tendon gel preserved *in vivo* for 10 days is an appropriate condition for regenerating tendon gel.

The diameter of the collagen fiber increased with increasing tensile stress. In this study, the diameter of the observed collagen fiber in the elongated tendon gel was approximately $10\text{ }\mu\text{m}$ after applying a tensile stress of approximately 9 MPa. An increase in the diameter of collagen fiber will contribute to improvement of the mechanical properties of a formed tendon analog. The qualitative understanding of the regenerative process of tendon gel was advanced in this study. However, further investigation on the quantitative relation between the magnitude of tension application for tendon gel and the time will be required using larger animals to produce an artificial tendon. The investigation of the regeneration mechanism of the tendon gel will lead to useful information to solve the clinical challenge on tendon repair. Interestingly, the regenerative structure of tendon gel is

similar to the fundamental structure of the ligament [41, 42], and the production technique of the regenerative biomaterial described in this research could an option for a future strategy to treat ligament injuries as well as tendon injuries.

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Compliance with ethical standards

Conflicts of interest The authors have no financial conflicts of interest.

Ethical approval Animal care and procedures were approved and performed in accordance with the standards set forth by the Tokai University Animal Care and Use Committee (Approval Numbers: 143031, 154004).

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