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# Development of fibrocartilage layers in Achilles tendon enthesis in rabbits

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## Abstract

**Objective:** The details regarding the development of fibrocartilage layers in Achilles tendon (AT) enthesis are unknown. Therefore, we evaluated the development of fibrocartilage layers in AT enthesis using a rabbit model.

**Materials and Methods:** Forty-eight male Japanese white rabbits were used in this study. Six of them were euthanized at different stages (day 1, and 1, 2, 4, 6, 8, 12, and 24 weeks of age). The proliferation, apoptosis, Sox9-positivity rates, and chondrocyte number were evaluated. Additionally, safranin O-stained glycosaminoglycan (GAG) areas, width of AT enthesis, and calcaneus length were assessed. All parameters were compared to those at 24 weeks of age.

**Results:** The level of chondrocyte apoptosis was high from 1 to 8 weeks of age, and high expression level of Sox9 was maintained from day 1 to 6 weeks of age, which decreased gradually. Safranin O-stained GAG areas increased up to 12 weeks, calcaneus length increased up to 6 weeks, and the width of AT enthesis increased up to 1 week of age.

**Conclusion:** The changes in chondrocyte and extracellular matrix were completed by 8 and 12 weeks of age, respectively. The development of fibrocartilage layers in AT enthesis was completed by 12 weeks of age. Our results contribute to the administration of appropriate treatments based on age and aid in the development of novel methods for regenerating AT enthesis.

**Key words:** Achilles tendon enthesis, chondrocyte, fibrocartilage development, fibrocartilage layer, glycosaminoglycan

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## Introduction

Direct-type attachments, such as Achilles tendon (AT) enthesis, quadriceps tendon (QT) insertion, patellar tendon (PT) insertion, and anterior cruciate ligament (ACL) insertion, involve four transitional tissue layers: ligaments or tendons, two fibrocartilage layers (unmineralized and mineralized), and bone[1]. The varying degree of stiffness in these layers reduces the concentration of stress at the insertion site[1]. Glycosaminoglycans (GAGs) in the fibrocartilage layers contribute to the tissue elasticity[2], which confers resistance to tensile, shear, and compressive stresses, and thus, they are important for load transmission[1, 2].

To examine the development of fibrocartilage layers during ACL, QT, and PT insertions, quantitative morphometric evaluations have been performed in rabbits[3, 4]. During the development of fibrocartilage layers during tibial ACL insertion in rabbits, chondrocyte proliferation and sex-determining region Y box 9 (Sox9) expression increases until 12 weeks of age, along with GAG production, which is in accordance with the increase in ACL length and its insertion width[3]. The development of fibrocartilage layers during ACL insertion is completed by 12 weeks of age[3]. In rabbits, the development of fibrocartilage layers during QT and PT insertions is also completed by 12 weeks of age[4]. However, the developmental processes of fibrocartilage layers during QT and PT insertions differ substantially in rabbits[4].

In a previous study, qualitative evaluations revealed the histological developmental changes during AT enthesis in humans[5]. In this study, the appearance of each tissue was revealed during the enthesis of each organ[5]. However, the development of fibrocartilage layers during AT enthesis has not been quantitatively evaluated. The knowledge regarding the normal anatomical structure is required for optimal treatment and rehabilitation. Moreover, specific treatments

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targeting the development of the normal structure during AT enthesis at all ages are required. Therefore, further studies are needed to evaluate the formation process and anatomical structural differences in the growth of fibrocartilage layers during AT enthesis.

In this study, we evaluated the development of fibrocartilage layers during AT enthesis using a rabbit model and performed quantitative morphometric evaluations.

## Materials and Methods

### Animal preparation

Forty-eight male Japanese white rabbits were used in this study. As the skeletal growth of rabbits is completed by 6 months of age[6], we considered the final evaluation time point as 24 weeks of age. Since we aimed to evaluate the development of fibrocartilage layers during AT enthesis in this study, *in vitro* experiments were not feasible to be performed. We used rabbits as model animals because it is difficult to prepare tissue specimens from smaller animals. Rabbits were maintained in accordance with the guidelines of our institution's Ethical Committee and National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23 Rev. 1985). Six animals each (at days 1 and 1, 2, 4, 6, 8, 12, and 24 weeks of age) were euthanized through intravenous injection of an overdose of barbiturate (200 mg/kg, Somnopentyl®, Kyoritsu Seiyaku Corporation, Tokyo, Japan).

### Histomorphological analysis

AT and calcaneus complex harvested from the animals were fixed using 10% neutral-buffered formalin for 1 week. After fixation, the specimens collected from rabbits at 2 to 24 weeks of age were decalcified using 10% ethylenediaminetetraacetic acid (pH 7.4) for 7 to 12 weeks, and then embedded in paraffin. The specimens from rabbits at day 1 and 1 week of age were sliced without decalcification. The collected specimens were sliced into sagittal sections of 5 µm thickness at the center of AT enthesis. The slices were stained with hematoxylin and eosin (H&E) and safranin O to assess the histomorphological characteristics and GAG content[3, 4, 7–10]. We also performed the proliferating cell nuclear antigen (PCNA) staining to detect the proliferating cells (Figure 1a)[3, 4, 7–10], terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) staining to detect the apoptotic cells (Figure 1b)[3, 4, 7–10], and Sox9 staining to evaluate the developmental differentiation of chondrocytes (Figure 1c)[3, 4]. For PCNA staining, the Histofine® SAB-PO (M) kit (Nichirei Biosciences, Inc., Tokyo, Japan) was used according to the manufacturer's instructions[3, 4, 7–10]. The anti-PCNA monoclonal antibody (PC-10; Code No. M0879; Dako, Glostrup, Denmark) and antibody diluent (Code No. S0809; Dako) have also been used[3, 4, 7–10]. For TUNEL staining, the Apoptag® Plus Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore, Billerica, MA, USA) was used following the manufacturer's instructions[3, 4, 7–10]. For Sox9 staining, the Histofine® SAB-PO (R) Kit (Nichirei Biosciences, Inc.) and Rabbit-To-Rabbit blocking reagent (ScyTek Laboratories, Inc., Logan, UT, USA) were used according to the manufacturer's instructions[3, 4].

The histomorphometric analyses were performed using the methods similar to those used in our previous study[3, 4, 7–10]. The sections were examined using the BX-51 light microscope (Olympus, Tokyo, Japan). The GAG regions stained red through safranin O were observed in the fibrocartilage layers in AT enthesis (Figure 2). In the specimens harvested from rabbits at day 1, and 1 and 2 weeks of age, we characterized the fibrocartilage layers during AT enthesis as those with the staining intensity lower than that of the articular cartilage using safranin O with round cells, and were found between the ligament and hyaline cartilage area in continuation with the articular cartilage. In specimens of rabbits >4 weeks of age, we defined the fibrocartilage layers in AT enthesis as the cartilaginous tissue with round cells present between the tendon and bone[3, 4]. The width of AT enthesis was defined as the proximal-to-distal distance of the AT calcaneus attachment. The width of AT enthesis and vertical width of calcaneus were measured. Mac Scope software (Mitani Co., Fukii, Japan) was used to determine the total number of chondrocytes and number of TUNEL-positive, PCNA-positive, and Sox9-positive chondrocytes in the safranin O-stained GAG areas present in the fibrocartilage layers of AT enthesis. Each red-stained GAG area and tidemark length were divided by the width of AT enthesis to determine the average thickness of the red-stained GAG areas. The tidemark length in AT enthesis was evaluated as the sum total length stained with H&E. The number of TUNEL-, PCNA-, and Sox9-positive chondrocytes were calculated based on the total number of chondrocytes in the safranin O-stained GAG areas in the fibrocartilage layers.

### Statistical analysis

For each parameter, the data was normalized using the Shapiro-Wilk normality test. The time-dependent histological changes were evaluated using the one-way analysis of variance (ANOVA) when all variables for each parameter were normalized. The factors that exhibited significant differences in the analysis of variance were further evaluated using the Dunnett's test. When the assumption of normality failed for all variables for any parameter, the Kruskal-Wallis test and Bonferroni adjustment were applied. The values of all parameters were compared to those at 24 weeks of age. The level of significance was set at 5%. All analyses were performed using the SPSS version 26.0 software (IBM Corp., Armonk, NY, USA).

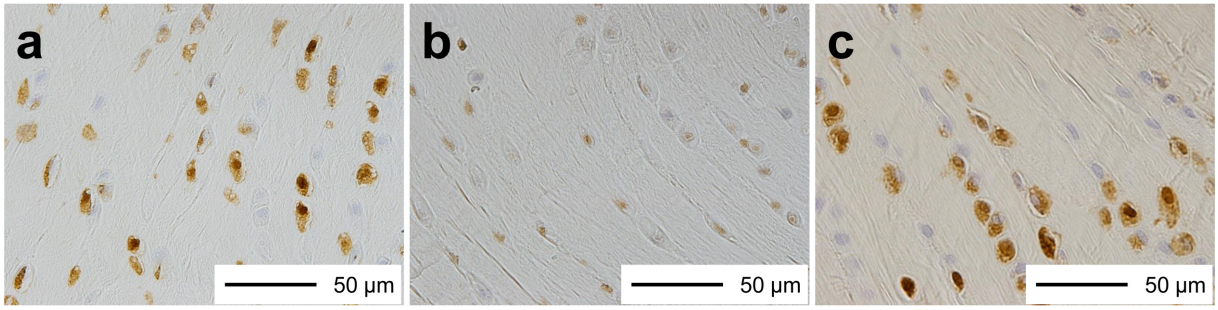


**Figure 1** Immunohistochemical staining. (a) PCNA staining (400×). PCNA-positive chondrocytes are shown in brown. (b) TUNEL staining (400×). TUNEL-positive chondrocytes are shown in brown. (c) Sox9 staining (400×). Sox9-positive chondrocytes are shown in brown. PCNA, proliferating cell nuclear antigen; TUNEL: terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling; Sox9: sex-determining region Y box 9.

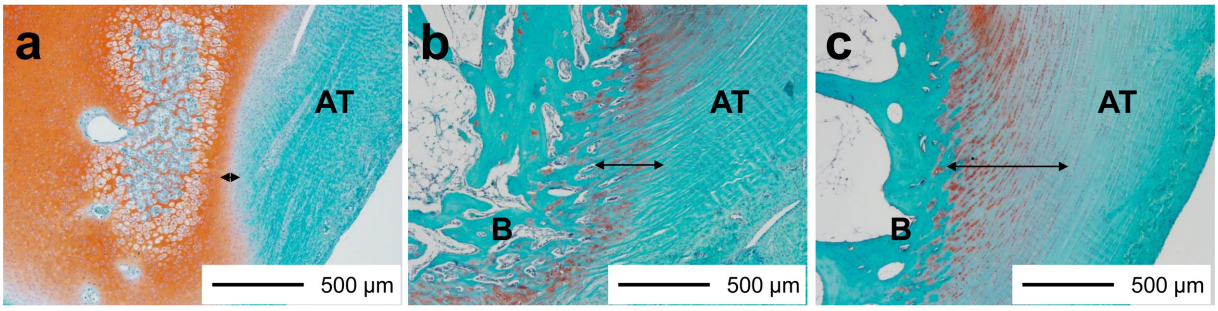


**Figure 2** Histological sections of AT enthesis stained with safranin O for each age group. The interfacial fibrocartilage layers are shown using black arrows. Images of rabbit specimens collected at (a) 2, (b) 8, and (c) 24 weeks of age (40×) are shown. AT: Achilles tendon; B: bone.

Based on a previous study[3, 4], the power calculations were performed with a confidence level of 95% (α=0.05), and power (1 - β) of 80% using the POWER Procedure in SAS software (SAS Institute, Cary, NC, USA). The evaluation of the smallest sample size to produce a significant difference yielded an estimated sample size of five to six specimens per age group. Therefore, we included six specimens for each age group to minimize the number of animals used.

## Results

The histomorphometric findings are summarized in Table 1. The chondrocyte proliferation rates from day 1 to 24 weeks of age were not significantly different from that at 24 weeks of age. The chondrocyte apoptosis rates at 1, 6, and 8 weeks of age were significantly higher than that at 24 weeks of age. The number of Sox9-positive chondrocytes at day 1 and 1, 2, 4, and 6 weeks of age were significantly higher than that at 24 weeks of age. The number of chondrocytes from day 1 to 24 weeks of age was not significantly different from that at 24 weeks of age. The thicknesses of safranin O-stained GAG areas at day 1, and 1, 4, 6, and 8 weeks of age were significantly lower than that at 24 weeks of age. The width of AT enthesis at day 1 of age was significantly lower than that at 24 weeks of age. The vertical widths of the calcaneus at day 1, and 1, 2, and 4 weeks of age were significantly lower than that at 24 weeks of age.

## Discussion

High degree of chondrocyte apoptosis was observed from 1 to 8 weeks of age, whereas Sox9 expression was high from day 1 to 6 weeks of age, after which both parameters decreased gradually. Safranin O-stained GAG areas increased for up to 12 weeks of age, and the width of AT enthesis increased for up to 1 week of age. Therefore, the development of fibrocartilage layers in AT enthesis completed by the age of 12 weeks.

An increase in the level of chondrocyte apoptosis was observed until 8 weeks of age. Although chondrocyte proliferation did not change, high expression level of Sox9 was observed until 6 weeks of age, after which it decreased. During ACL insertion in rabbits, chondrocyte proliferation and Sox9 expression were high until 4 and 8 weeks of age, respectively, and then decreased gradually[3]. High level of chondrocyte apoptosis was maintained until 8 weeks of

**Table 1** Results of histomorphometric analyses

| | 1 d ($n$ = 6) | 1 w ($n$ = 6) | 2 w ($n$ = 6) | 4 w ($n$ = 6) | 6 w ($n$ = 6) | 8 w ($n$ = 6) | 12 w ($n$ = 6) | 24 w ($n$ = 6) | partial $\eta^2$ / power |
|---|---|---|---|---|---|---|---|---|---|
| Chondrocyte proliferation rate (%) | 52.6 ± 20.4 | 50.2 ± 15.3 | 34.8 ± 3.2 | 32.5 ± 11.8 | 50.6 ± 17.4 | 58.4 (40.8–71.1) | 45.3 ± 6.5 | 44.6 ± 3.3 | 0.301 / 0.805 |
| Chondrocyte apoptosis rate (%) | 44.6 ± 14.4 | 56.6 ± 11.9* | 30.7 ± 6.7 | 46.8 ± 19.8 | 55.8 ± 7.6* | 67.9 (47.8–77.8)* | 45.1 ± 4.2 | 42.1 (37.8–44.2) | 0.447 / 0.984 |
| Sox9-positive chondrocyte rate (%) | 49.6 (42.1–52.3)* | 37.5 ± 5.2* | 36.8 ± 5.1* | 32.4 ± 3.0* | 29.7 ± 2.7* | 27.9 ± 4.3 | 12.8 ± 3.9 | 8.3 ± 2.3 | 0.914 / 1.000 |
| Number of chondrocytes / width of enthesis (n/mm) | 192.7 (124.8–338.6) | 190.8 ± 76.0 | 265.6 ± 89.8 | 289.8 ± 111.9 | 220.6 ± 61.6 | 223.5 ± 76.1 | 177.2 ± 18.2 | 161.4 ± 33.4 | 0.249 / 0.675 |
| Thickness of safranin O-stained glycosaminoglycan areas (μm) | 74.6 ± 35.9* | 105.1 ± 33.9* | 118.3 ± 31.8 | 97.3 ± 32.1* | 60.1 ± 6.2* | 46.3 (26.7–118.4)* | 169.5 ± 51.2 | 197.8 ± 59.2 | 0.614 / 1.000 |
| Width of enthesis (mm) | 0.60 ± 0.11* | 0.87 ± 0.17 | 0.88 ± 0.25 | 1.11 ± 0.34 | 1.27 ± 0.18 | 1.31 ± 0.16 | 1.24 ± 0.18 | 1.17 ± 0.22 | 0.597 / 1.000 |
| Vertical width of the calcaneus (mm) | 1.78 ± 0.20* | 2.82 ± 0.33* | 3.46 ± 0.66* | 3.94 ± 1.01* | 4.96 (4.57–5.16) | 5.26 ± 0.54 | 5.48 ± 1.01 | 6.21 ± 0.79 | 0.838 /1.000 |

Results are presented as the mean ± standard deviation, if normality was confirmed. Results are presented as median (interquartile range), if normality was not confirmed. *$P$<0.05, compared with the value at 24 weeks of age. d: day; w: week.

age[3]. During QT insertion in rabbits, low levels of chondrocyte proliferation were maintained until 2 weeks of age[4]. During PT insertion in rabbits, low levels of chondrocyte proliferation were maintained until 1 week of age, and high levels of chondrocyte apoptosis were observed at 1 day of age[4]. Low expression level of Sox9 expression was maintained until 1 week of age[4]. Although the behavior of chondrocytes differed towards AT, ACL, QT, and PT, differences in the mechanical environment, such as muscle strength and load, might have had an effect. These differences in chondrocyte behavior may be related to the differences in mechanical environments, such as musculoskeletal growth and gait, and/or differences in morphology and location.

At 12 weeks of age, the enthesis width, calcaneus length, and GAG production increased in AT enthesis. For the growth process of the extracellular matrix in the insertions and skeletons, AT, ACL, QT, and PT exhibited similar changes[3, 4]. The increase in safranin O-stained GAG areas might be due to mechanical stress. In accordance with gait and skeletal growth, these insertions and entheses undergo tensile, shear, and compressive stresses. Such mechanical stresses are important for the development of fibrocartilage layers during insertion and enthesis[7, 10–12]. GAGs, which contribute to tissue hydration and elasticity, also provide resistance against mechanical stresses[1]. Therefore, the increase in GAG production may be associated with the increased mechanical stress at the insertion and enthesis sites.

Sport-associated enthesopathies are observed in AT enthesis[11]. Moreover, bony spurs (enthesophytes) are well documented in AT enthesis[11]. Bony spurs become more common with increasing age[11]. Understanding the differences in the anatomical structure accompanying the growth of fibrocartilage layers in AT enthesis may contribute to the development of an appropriate treatment strategy based on age.

There were a few limitations in this study. As the skeletal growth of rabbits was complete[6], we performed quantitative analyses for only up to 6 months of age. However, the evaluations after 6 months might have been necessary for examining the fibrocartilage layer after the growth period. Moreover, mechanical analyses are also necessary to conduct to further clarify the association of fibrocartilage layer with mechanical stresses.

## Conclusion

Overall, we found that in a rabbit model of AT enthesis development, the changes in chondrocyte and extracellular matrix are completed by 8 and 12 weeks of age, respectively. The development of fibrocartilage layers in AT enthesis was completed by 12 weeks of age. Our results contribute to the administration of appropriate treatments based on the age, and aid in the development of methods for regenerating AT enthesis.

**Authors' contributions:** All authors conceived the study and participated in its design and coordination. All authors performed the animal experiments. All authors performed the histomorphometric analyses. Hirotaka Mutsuzaki analyzed the data and drafted the manuscript. All authors interpreted the data and participated in drafting the text and tables. All authors have read and approved the final version of the manuscript.

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**Conflicts of interest:** The authors declare that they have no conflicts of interest.

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