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Original article

Histological differences in cartilage layer growth at various tendon and ligament insertions in rabbits

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Abstract

Objectives: Histological differences in cartilage layer growth in Achilles tendon (AT), quadriceps tendon (QT), patellar tendon (PT), and anterior cruciate ligament (ACL) insertion are unclear. Therefore, this study aimed to investigate the differences in cartilage layer growth in AT, QT, PT, and ACL insertions.

Materials and Methods: Forty-eight male Japanese white rabbits were used. Six animals were euthanized at different stages (day 1 and 1, 2, 4, 6, 8, 12, and 24 weeks). Safranin O-stained glycosaminoglycan (GAG) production area, chondrocyte count, and insertion width were investigated.

Results: A two-way analysis of variance (ANOVA) revealed a significant difference in the main effects of time and insertion for all parameters. In addition, the time × insertion interaction was significant. Multiple comparisons showed a significant difference between the ACL insertion and all other variables; however, the GAG production area was not significantly different for the QT, PT, and AT insertions. AT insertions were significantly different from all other groups; however, the number of chondrocytes and insertion width were not significantly different for ACL, QT, and PT insertions.

Conclusion: Cartilage layer growth differed between the AT, QT, PT, and ACL insertions. The differences between the insertions may also be due to the differences in their structures, locations, and mechanical environments.

Keywords: insertion, fibrocartilage layers, growth, tendon, ligament

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Introduction

Tendon and ligament insertions, including the Achilles tendon (AT), quadriceps tendon (QT), patellar tendon (PT), and anterior cruciate ligament (ACL) insertions, have four transitional tissue layers: tendon/ligament, unmineralized fibrocartilage, mineralized fibrocartilage, and bone[1]. The different stiffness values of the tissue layers reduced the stress concentration at the insertion site[1]. Glycosaminoglycans (GAGs) in the fibrocartilage layer of tendons and ligamentous insertions provide tissue hydration and elasticity and mainly resist tensile and shear stresses[1–4]. Thus, the GAG production layer plays an important role in load transmission at the tendon/ligament insertion sites. These four insertions, categorized as direct insertions, showed similar formations, including two cartilage layers. However, their locations, surrounding structures, and mechanical environments differ. Specifically, both ends of the ACL are fixed to the bone, whereas one end is fixed to the bone and the other end is attached to the muscle in the QT, PT, and AT (with the PT having a muscular end via the patella).

AT, QT, PT, and ACL tibial insertions grow in up to 24-week-old rabbits[5–7]. Fibrocartilage layer growth in these insertions is completed in 24 weeks of age[5–7]. However, the periods of high chondrocyte count and thicker GAG in QT insertion were longer than those in PT insertion, up to 12 weeks[6]. These differences can be attributed to the distinct mechanical stresses experienced by these structures. The QT insertion is directly connected to the quadriceps muscle and may be exposed to a more tensile environment than the PT insertion[6]. Moreover, the differences in fibrocartilage

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layer growth in AT, QT, PT, and ACL tibial insertions are unclear. Furthermore, because the locations, surrounding structures, and mechanical environments of the AT, QT, PT, and ACL tibial insertions differ, their development processes may vary.

Therefore, this study investigated the differences in the fibrocartilage layer growth in AT, QT, PT, and ACL tibial insertions in rabbits. We hypothesized that the development of fibrocartilage layers would differ among these insertions in rabbits.

Clinically, several injuries occur at the AT, QT, PT, and ACL tibial insertion sites. Examples include Achilles tendinopathy[8], and Achilles tendon rupture[9] in AT injuries, jumper's knee in QT patellar tendinopathies[10], and ACL injury, which is one of the most well-known injuries among athletes[11]. Understanding the differences in the growth process of each insertion suggests that treatment strategies may differ depending on the growth period of each insertion.

Materials and Methods

Animal preparation

The animal species were determined based on previous reports[5–7]. We chose rabbits for this study because this research could not be undertaken *in vitro* and preparing tissue specimens using animals smaller than rabbits is challenging. Forty-eight male Japanese white rabbits were used in this study. On day 1 and at 1, 2, 4, 6, 8, 12, and 24 weeks of age, six rabbits in each group were euthanized by intravenously injecting 200 mg/kg barbiturate (Somnopentyl®, Kyoritsu Seiyaku Corporation, Tokyo, Japan). At six months, the skeletal growth of the rabbits was complete[12]; therefore, the final investigation period was set at 24 weeks.

The rabbits were maintained in accordance with the guidelines of the Institutional Ethical Committee and the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23, rev. 1985). This study conformed to the Animal Research: Reporting In Vivo (ARRIVE) guidelines.

Staining method and immunohistochemistry

Specimens were fixed with 10% neutral-buffered formalin, decalcified, and embedded in paraffin, according to our previous reports[5–7]. Decalcification was not required for rabbit specimens at 1 d or 1 week of age. All unilateral AT and calcaneus complex, QT–patella–PT complex, and knee specimens were sliced at 5 μm in the sagittal plane[5–7].

Hematoxylin and eosin and safranin O staining were performed to evaluate the histomorphology and GAG production[5–7].

Histomorphometric analysis

Histomorphometric analysis was performed on each glass slide by a single examiner, in accordance with our previous report[5–7]. For each specimen, 5-μm-thick sections from the sagittal plane at the center of the insertion site were stained with safranin O. In the fibrocartilage layers of the AT calcaneus, PT patella, QT patella, and ACL tibial insertions, the safranin O-stained regions were identified as GAG production areas (Figure 1)[5–7]. The widths of the AT calcaneus, QT patella, PT patella, and ACL tibial insertions were measured at the safranin O-stained fibrocartilage layers[5–7]. The chondrocyte counts and GAG production areas were observed and measured using a BX-51 light microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and MacScope software (Mitani Co., Fukui, Japan)[5–7]. The total chondrocyte count and safranin O-stained GAG production areas was divided by the width of each insertion[5–7]. Subsequently, the number of chondrocytes per insertion width and GAG production area thickness were calculated[5–7] and compared between groups.

Statistical analysis

For each parameter, a two-way analysis of variance (ANOVA) was used to analyze the main effects of time, insertion, and the interaction between time and insertion. Multiple comparisons were made for each parameter. $P<0.05$ was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics version 28.0 (IBM Corp., Armonk, NY, USA).

Power analysis was conducted using the POWER proce-

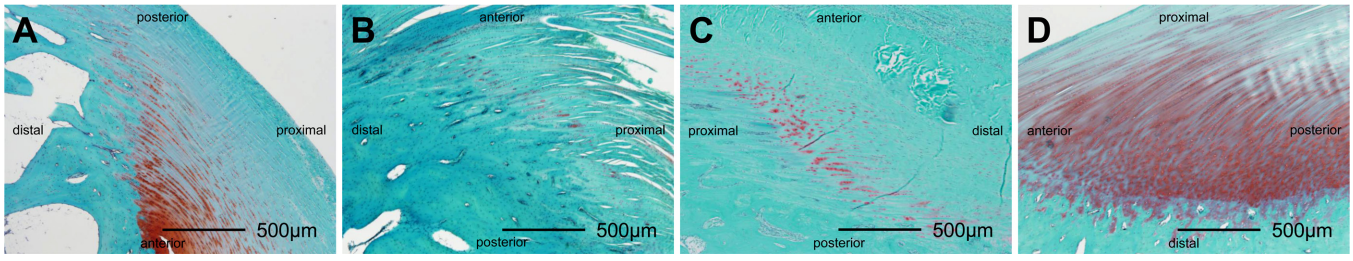

Figure 1 Safranin O-stained tissue specimens; the red area represents the glycosaminoglycan production area (40×). (A) Achilles tendon (AT), (B) quadriceps tendon (QT), (C) patellar tendon (PT), and (D) anterior cruciate ligament (ACL) tibial insertion at 24 weeks of age.

dure in SAS software (SAS Institute, Cary, NC, USA), with 95% confidence level (α=0.05) and 80% power (1–β), based on previous research[5–7]. The smallest sample size was 5–6 specimens per age group. Therefore, six specimens from each age group were included in this study.

Results

Two-way ANOVA revealed a significant difference in the main effects of time and insertion on GAG production area, chondrocyte count, and insertion width (P<0.001, Figures 2–4). Additionally, the time × insertion interaction was also significant for all parameters (P<0.001). Multiple comparisons revealed a significant difference in GAG production area and chondrocyte count between ACL and all other muscle insertions and AT and all other muscle insertions, respectively (P<0.001); moreover, AT insertion width was significantly different from that in the QT (P=0.006), PT (P=0.016), and ACL (P=0.001) insertions. However, GAG production area was not significantly different between the

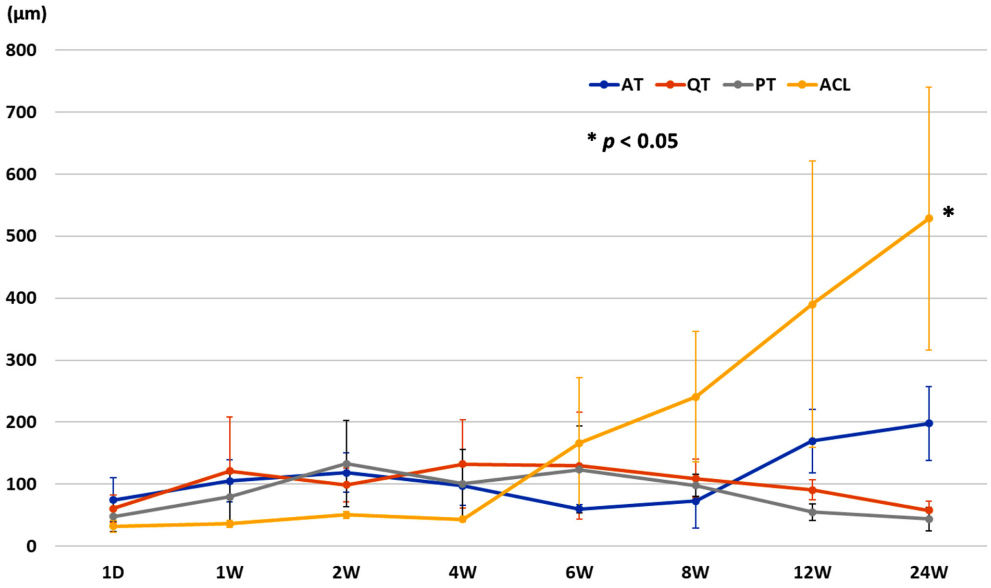

Figure 2 Growth in the glycosaminoglycan (GAG) at each insertion.
AT: Achilles tendon; QT: quadriceps tendon; PT: patellar tendon; ACL: anterior cruciate ligament.

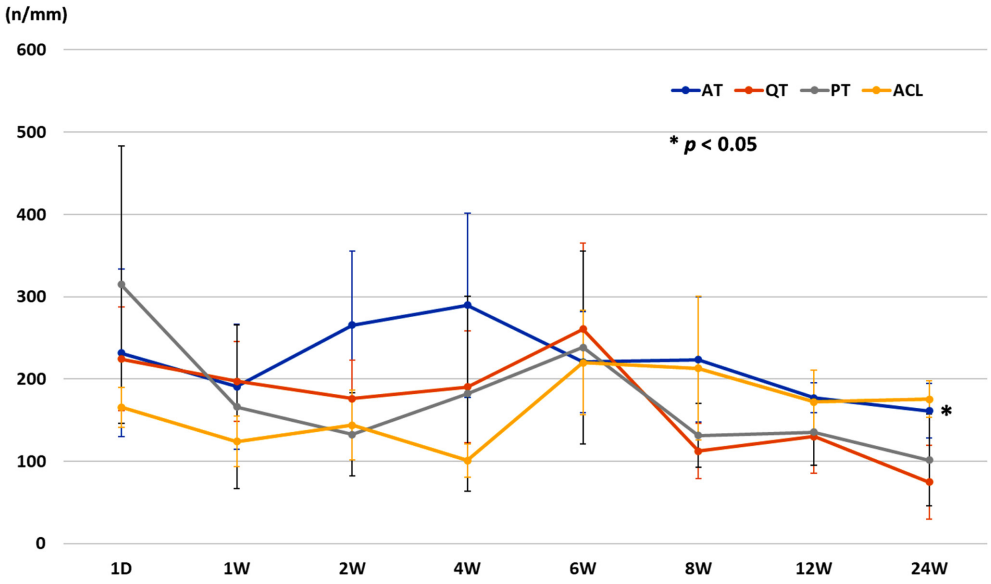

Figure 3 Growth in the number of chondrocytes at each insertion.
AT: Achilles tendon; QT: quadriceps tendon; PT: patellar tendon; ACL: anterior cruciate ligament.

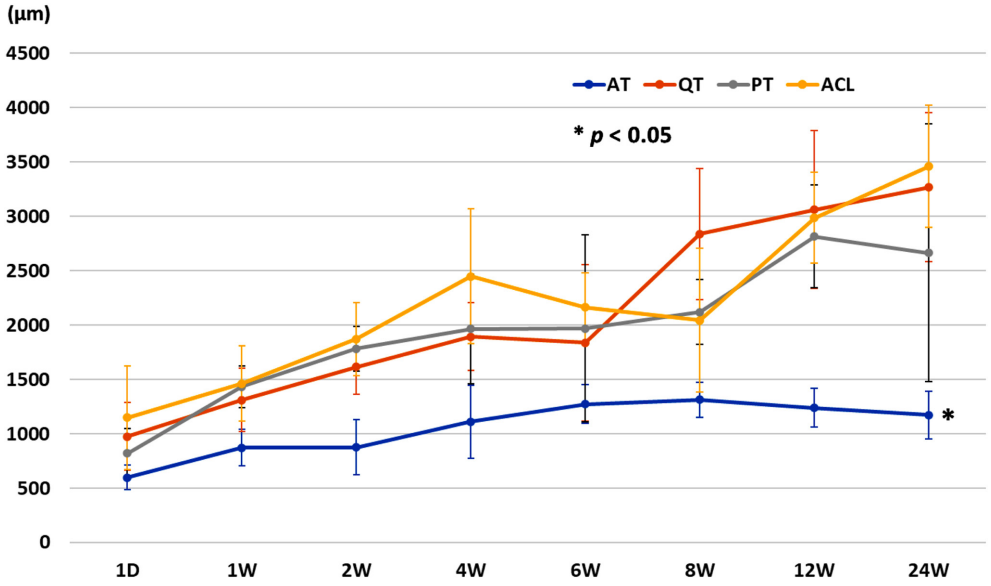

Figure 4 Growth in the width of each insertion.
AT: Achilles tendon; QT: quadriceps tendon; PT: patellar tendon; ACL: anterior cruciate ligament.

QT, PT, and AT insertions, while chondrocyte count and insertion width were not significantly different between the ACL, QT, and PT insertions.

Discussion

Even though the four insertions were classified as having the same direct insertion, GAG width, chondrocyte count, and insertion width were different in the cartilage layers of the AT, QT, PT, and ACL tibial insertions during the development process.

Mechanical stimulation such as tensile force increases cartilage layer thickness at the tendon–ligament insertion site. Gradual PT insertion elongation using external fixation significantly increased the average thickness of the stained GAG production areas in the cartilage layer compared with that in the sham group rabbits at 4 weeks[13]. Moreover, previous animal experiments using an ACL partial resection model have reported that the average stained GAG production area thickness increased at 2–4 weeks in the remaining ligament area than that in the resection area[14]. In contrast, the GAG layer thickness at the ACL tibial insertion decreased in an ACL-resection animal model[15, 16]. Moreover, mechanical unloading decreases the GAG layer thickness after PT insertion in rabbits for up to six weeks[17]. Furthermore, GAG layer thickness in the ACL tibial insertion in the immobilized rabbits was lower than that in the sham group rabbits 2–8 weeks after surgery[18]. Therefore, GAG layer thickness can be affected by mechanical stress. The ACL insertion may receive a high load as it matures. This may be the reason for the increase in GAG in the ACL tibial insertion than that in other insertions.

The differences between the AT, QT, PT, and ACL tibial insertions can also be attributed to the differences in their structures, locations, and mechanical environments. ACL has two bone ends, whereas the AT, QT, and PT complexes have one muscle end. Muscle tension and traction during the growth period affect the AT, QT, and PT complexes. Locations around the ankle or knee joints may also influence differences in growth processes. This may have influenced the change in chondrocyte count and insertion site width at the AT. Till date, no studies have directly or comprehensively examined the mechanical aspects of AT, QT, PT, and ACL insertion in rabbits. Furthermore, no reports have evaluated the mechanical properties of the cartilage layer in rabbits. A previous study has showed that the modulus of the PT was 89% higher than that of the ACL[19] and the failure load for the PT was greater than that for the ACL in rabbits[20]. However, these studies considered the tendon/ligament–bone complex as a single structure and did not evaluate only the cartilaginous layers. Moreover, because the cross-sectional areas of the tendon and ligament were not considered, the load of the PT, which has a large cross-sectional area, was larger than the load of the ACL, which has a small cross-sectional area. Therefore, the mechanical load per unit cross-sectional area is unknown, and further studies are warranted to investigate the mechanical load per unit cross-sectional area. Conversely, the ultimate failure load per unit cross-sectional area of the AT increases with maturation in rabbits[21]. Therefore, the mechanical load per unit cross-sectional area increases with growth.

Clinically, our results support the consideration of ap-

propriate treatment strategies based on the insertion site and the development of new treatments for the regeneration of the tendon–bone interface and insertions. In addition, the initial tension and fixation materials should be considered according to the age and location when repairing the interface.

This study has some limitations. Although complete skeletal growth in rabbits is achieved at six months of age[12], further evaluation may be necessary to investigate the effects of growth. Moreover, mechanical evaluation is necessary to elucidate the influence of mechanical loads.

Conclusions

Cartilage layer growth differed after AT, QT, PT, and ACL tibial insertions. This may also be due to differences in structures, locations, and mechanical environments.

Conflicts of interest: The authors declare no conflict of interest.

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Institutional review board statement: The animal research protocol was approved by the Ethics Committee of Ibaraki Prefectural University of Health Sciences. The rabbits were maintained in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23, rev. 1985).

Data availability statement: The datasets used and/or analyzed in this study are available from the corresponding author upon reasonable request.

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