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Histopathological study of the infrapatellar fat pad in the rat model of patellar tendinopathy: A basic study

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ABSTRACT

Background: Patellar tendinopathy is difficult to successfully treat. This study aimed to characterize the pathological changes of the infrapatellar fat pad (IPFP) in patellar tendinopathy (PT), and to investigate the influence of PT on the development of fibrotic changes in the IPFP.
Methods: Forty male Wistar rats were randomly divided into PT (n = 20) and control groups (n = 20). Bacterial collagenase I (patellar tendinopathy group) or saline (control) was injected, intratendinous, into the patellar tendon. Rats were sacrificed at week 12. The whole knee joint was sagittally sectioned and stained with hematoxylin–eosin and Masson's trichrome. The IPFP samples were graded according to cellularity, fibrosis, and vascularity. The whole IPFP and blue-stained area was measured. Mann–Whitney *U* tests were used to compare the between-group differences of each score and quantitative value.
Results: Scores for cellularity were three (2–3) and 0 (0–1) in the PT and control groups, respectively ($P < 0.01$). Mean scores for fibrosis were two (1–3) and 0 (0–1) in the PT and control groups, respectively ($P < 0.01$). Mean scores for vascularity were two (2–3) and one (1–1) in the PT and control groups, respectively ($P < 0.01$). There was a significant difference in the total score between the PT and control groups (seven (5–8) and two (1–3), respectively) ($P < 0.01$). Average percentages of the fibrous area of the IPFP were 38.2 ± 26.5% and 11.2 ± 3.9% in the patellar tendinopathy and control groups, respectively ($P < 0.01$).
Conclusion: The IPFP in the patellar tendinopathy group showed greater cellularity, fibrosis, and vascularity than the control group.

1. Introduction

The infrapatellar fat pad (IPFP) is an intra-capsular, but extrasynovial, structure posteriorly covered by the synovial membrane [1]. The IPFP is thought to play both a biomechanical role in the patellofemoral joint and an inflammatory role in the knee joint by secreting pro-inflammatory or anti-inflammatory cytokines [2,3]. Biomechanically, the IPFP contributes to stabilizing the patella in knee motion [4]. It has not been demonstrated whether IPFP pathology results in idiopathic knee inflammation. Some inflammatory cells were observed in a collagenase-induced degenerative tendon injury model [5], and vascular connections between the

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IPFP and patellar tendon have been found [6]. However, details of the function and role of the IPFP in knee injury such as patellar tendinopathy (PT) have not been reported.

Patellar tendinopathy is primarily a condition that affects relatively young athletes. It is also common in athletes who participate in sports that require repetitive loading of the patellar tendon. The repetition of jumping and landing or insufficient rest to enable patellar tendon remodeling can induce pathological changes in the knee structure, which may cause anterior knee pain [7]. Lian et al. reported that the prevalence rates of patellar tendon overuse injury for basketball and volleyball players were up to 45% and 32%, respectively [8]. As the etiology and pathogenesis of PT remains unclear, it is a difficult condition to successfully treat.

Although PT is clinically caused by overuse of the patellar tendon [9], it has been induced by an intratendinous injection of bacterial collagenase in rats, according to a previous study [5]. The key features of tendinopathy include the following: hypercellularity; hypervascularity; ectopic chondrogenesis; sustained expressions of decorin, biglycan, fibromodulin, and aggrecan; and expressions of substance P and calcitonin gene-related peptide in the patellar tendon. The IPFP contains many arterial vessels to supply the patellar tendon [6], and this may contribute to PT via secretion of local biochemical mediators, including pro-inflammatory cytokines [10,11]. Adipose tissues are a source of cytokines and growth factors [12]. The IPFP might also cause an inflammatory response and express pro-inflammatory cytokines in those with PT. One study has already investigated the effect of adipose tissue on the Achilles tendon [13]. Thus, there might be some co-action between pathological changes in the IPFP and knee joint injury. Once pathological changes in the IPFP with PT have been evaluated, it will be beneficial to elucidate the association of pathogenesis and interaction between the IPFP and patellar tendon in PT. However, no previous studies have quantitatively characterized changes to the IPFP in response to PT.

The purposes of this study were to characterize the pathological changes in the IPFP with PT using a grading system and to quantitatively and histologically investigate the effect of PT on the development of fibrotic changes in the IPFP using a rat PT model. Considering the cross-sectional nature of the present study, PT was induced via an injection, since this method is highly reproducible [14]. Patellar tendinopathy is a common overuse injury, particularly in relatively young athletes (aged 15–30 years) [7]. Rats are considered adolescents from the eighth to ninth week of postnatal life [15]; therefore, 9-week-old rats were selected to match the tissue condition between the animal model and humans. It was hypothesized that the IPFP would exhibit increased cellularity, fibrosis, and vascularity in PT. It was also hypothesized that the amount of collagen fibers would increase in PT.

2. Materials and methods

2.1. Animal model and collagenase-induced injury

The Kanazawa University Animal Research Ethics Committee (approval no. AP-153629) approved use of rats for experiments in this study. In order to increase the number of samples in the present study, compared to previous studies [16,17], and because of limited facilities for husbandry of larger animals such as sheep, a PT rat model was used. Forty Wistar male rats (age: nine weeks; body mass: 228.1 ± 6.3 g) were used in this study. They were divided into two groups using block randomization: a PT group (n = 20) and control group (n = 20). After administering anesthesia with 2.5% pentobarbital (4.5 mg/kg body weight) intraperitoneally, the hairs over the lower limb were shaved, and the patellar tendon was located by positioning the knee at 90°. Twenty microliters (0.015 mg/ml in 0.9% saline) of bacterial collagenase I (Wako Pure Chemical Industries Ltd., Osaka, Japan) (PT group) or saline (Otsuka Pharmaceutical Co., Ltd., Naruto, Japan) (control group) were injected, by T. K., intratendinous into the patellar tendon using a 30-gauge needle in the right limb, while the contralateral limb was left untreated [5]. The same person injected all animals on two separate days after the pilot trials. The dosing of collagenase was determined according

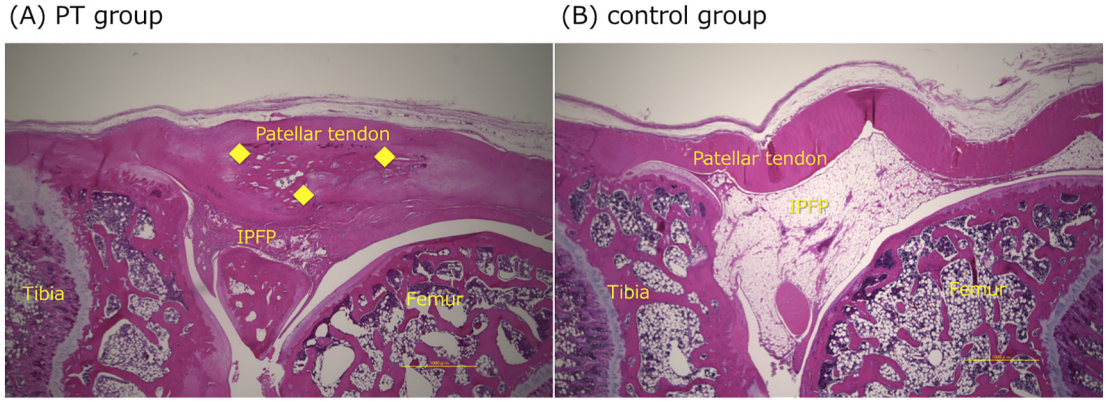

Figure 1. Sagittal sections of the anterior part of the knee joint with hematoxylin–eosin staining (10× magnification was used for analyzing, but images with 2× magnification were included so readers can see the entire IPFP). In the PT group, increased cellularity, fibrosis, and vascularity were observed in the IPFP. The tendon was thickened with the presence of ectopic calcification or ossification. PT, patellar tendinopathy; IPFP, infrapatellar fat pad; diamond, calcification or ossification.

to that used in previous studies [5,18]. The person who injected the rats in either group was not blinded to the injection liquid because the color of solid collagenase is brown, which can be easily differentiated from saline. No leakage of liquid outside the patellar tendon was confirmed using a stain solution in a pilot study.

The rats in both groups were allowed cage-free activity with ample food and water after injection. The injected area and knee did not display any infection, edema, and redness on the knee of rats in either group. The collagenase injection did not appear to have adverse effects on the animals because all the rats treated with collagenase gained weight during the study period. All the animals survived until they were sacrificed. They were sacrificed using an intraperitoneal injection of an undiluted solution of pentobarbital. The rats were sacrificed at week 12 because in a previous study [5], calcific deposits were present in the patellar tendon of all samples at week 12, and this is a common key feature of tendinopathy caused by the intratendinous injection of collagenase [14].

2.2. Histological examination of the infrapatellar fat pad

The section was washed in phosphate-buffered saline, fixed in formalin and methanol, embedded in paraffin, cut longitudinally to two-micrometer-thick sections, and mounted on slides. After deparaffination, the sections were stained with hematoxylin eosin and Masson's trichrome. For each section, three images were analyzed by using a fluorescent microscope (BZ-9000, Keyence, Tokyo, Japan) at a magnification of 10×. The field of view was about one-third of the whole body of the IPFP, and the image resolution was 4080 × 3072 pixels. One hundred and twenty images were ultimately obtained since there were 40 samples from rats in both groups. In the PT group, ectopic calcification or ossification was observed in the patellar tendon (Figure 1). To further validate the tissue modifications that resulted from PT, a histological score was used to assess the key aspects of inflammation observed in the IPFP. Quantification of the histology of the IPFP followed the scoring protocol is listed in Table 1, according to a previous study [16]. The grading protocol was developed to quantify the histopathology of the IPFP [16,17]. The synovium was not considered when scoring the histology. The observers established between-day intratester reliability by scoring 10 images separated by seven days between measurements. The interobserver reliability was 0.87, and 95% confidence interval (CI) was 0.69–0.95 for two experienced assessors (K. S. and K. A.). One hundred and twenty stained images were graded by a third experienced assessor (Y. T.) in a blinded manner. The total histological score is the summation of cellularity, fibrosis, and vascularity.

2.3. Quantitative analysis of collagen fibers

Each section stained with Masson's trichrome was viewed with the same calibration and settings using the analysis software Image J (National Institute of Health, Bethesda, MD, USA) (Figure 2). For each section, two images were taken at a magnification of 2×. The field of view was about the whole body of the IPFP. Forty images were graded by the two trained observers (T. K. and Y. T.) in a blinded manner. The blue-stained area (indicating collagen fibers) of the image was enhanced using color split channel function and adjustment configuration with a fixed threshold level. The area of the whole IPFP and the blue-stained area were measured. The interobserver reliability was 0.96, and 95% CI was 0.84–0.99 for two experienced assessors (K. S. and K. A.). The final value was taken as the average of the two investigators' values. The average value of the percentage of blue-stained area in comparison with the whole IPFP area was calculated to quantitatively assess the severity of fibrosis.

2.4. Statistical analysis

Statistical analysis for this study was performed using the Statistical Package for the Social Sciences (SPSS) for Windows version 23.0 (SPSS Inc., Chicago, IL, USA). The sample size was decided upon based on similar reports [16,17]. Post hoc power analysis was performed, and the results showed that the power of the test was 0.7. Mann–Whitney *U* tests were used to compare the difference of each score and quantitative value between the PT and control groups. Significance was accepted at a level of $P < 0.05$.

Table 1
Histological scoring protocol for the infrapatellar fat pad.

Criterion	Score	Description
Cellularity	0	Scattered cells
	1	Focal clustering
	2	Diffuse increase
	3	Severe diffuse increase
Fibrosis	0	Absent (<10% of the image area)
	1	Mild (10–30% of the image area)
	2	Moderate (30–60% of the image area)
	3	Severe (>60% of the image area)
Vascularity	0	No vessels present
	1	1–2 vessels
	2	3–4 vessels
	3	≥ 5 vessels

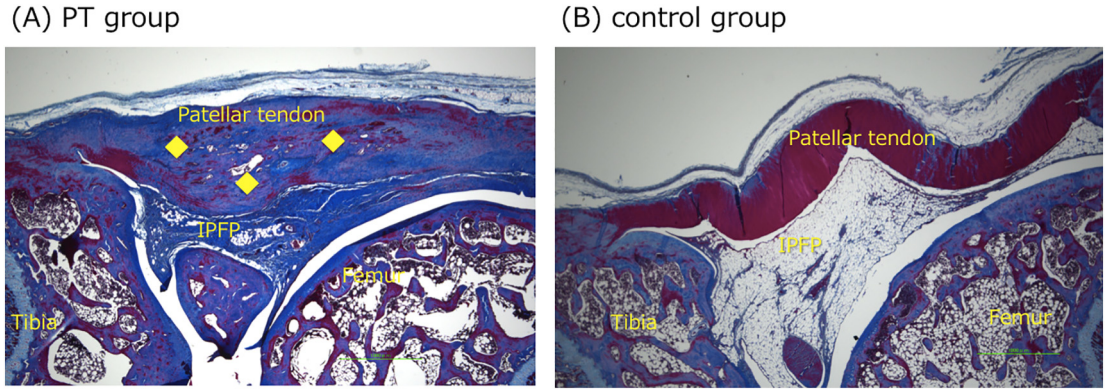

Figure 2. Sagittal sections of the anterior part of the knee joint with Masson's trichrome staining (2× magnification). The tendon was thickened with the presence of ectopic calcification or ossification in the PT group. PT, patellar tendinopathy; IPFP, infrapatellar fat pad; diamond, calcification or ossification.

3. Results

3.1. Patellar tendinopathy was established

As demonstrated in Figure 1, the IPFP tissue from the control rats demonstrated an even distribution of adipocytes with no evidence of fibrosis, indicating that overt inflammation was absent. Presence of calcification and/or chondrogenesis was confirmed in the patellar tendons of the PT group.

3.2. Histological results

The cellularity, fibrosis, vascularity, and total histological scores for the IPFP were significantly increased in the PT group compared with the control group (Table 2, $P < 0.01$). Masson's trichrome stains showed that the area of fibrotic change of the IPFP increased in the PT group (Figure 2). The fibrous area was statistically significantly greater in the PT group than in the control group. The average percentages of the fibrous area of the IPFP were $38.2 \pm 26.5\%$ in the PT group and $11.2 \pm 3.9\%$ in the control group ($P < 0.01$).

4. Discussion

Two purposes of this study were addressed: the histological changes of the rat IPFP in PT was characterized, and a fibrous change in the IPFP in PT was evaluated. The histology of the IPFP and severity of fibrosis were quantitatively evaluated in PT. In support of the study hypotheses, the findings of this study provide evidence that the IPFP tissue shows greater cellularity, fibrosis, and vascularity in PT. Other research has revealed histological changes, expression of inflammatory cytokines, and excessive synthesis of collagens in the IPFP after surgical anterior cruciate reconstruction surgery or surgically induced intraarticular drill injury in the repair process of tissue [16,17]. As a repair mechanism against PT, the IPFP might also cause some inflammatory response.

An increase of cellularity was observed in the IPFP of the PT group. Although immunostaining and messenger ribonucleic acid (mRNA) analysis were not performed in this study, this histological finding may indicate that cellularity increases due to an excessive increase of some types of collagen fibers in PT. To assess the potential molecular basis of this increased cellularity, the mRNA expression levels for some types of collagen and macrophage proteins need to be evaluated in further research.

The current study's results demonstrated histological evidence of fibrosis in the IPFP in response to PT. The underlying cause of this change might be due to up-regulation of transforming growth factor-β (TGF-β), platelet-derived growth factor, basic-fibroblast growth factor, or vascular endothelial growth factor (VEGF) mRNA expression [19,20]. Fibrosis results from an imbalance between excessive synthesis of fibrillar components and impairment in degradation of these proteins [21]. A clinical research study

Table 2
Histological grading of the infrapatellar fat pad.

	PT group	Control group	*P*
Cellularity score	3 (2–3)	0 (0–1)	<0.01
Fibrosis score	2 (1–3)	0 (0–1)	<0.01
Vascularity score	2 (2–3)	1 (1–1)	<0.01
Total score	7 (5–8)	2 (1–3)	<0.01

PT, patellar tendinopathy.

showed that fibrosis in the IPFP leads to activity-related pain or stiffness of the knee joint [22]. To reveal the development process of fibrosis of the IPFP in PT, further research using a longitudinal design is needed.

The present study showed angiogenesis of the IPFP in PT. TGF-β contributes to fibrosis of the IPFP, and it plays an important role in angiogenesis [23]. Although the present study could not evaluate the mRNA levels, angiogenesis might be caused by the levels of mRNA expression for molecules such as TGF-β, VEGF, and α-smooth muscle actin [16,24]. Angiogenesis is also essential for repair processes, but the proliferation of small vessels accompanying free nerve endings causes tissue fibrosis with a decreased threshold of pain sensation [24]. Some interventions to suppress excessive angiogenesis of the IPFP may help maintain the normal function of IPFP, both biomechanically and biochemically.

The present study had some limitations. First, the time-dependent histological change in the IPFP could not be observed because this study had a cross-sectional design. A longitudinal study will help determine when physicians should intervene to prevent irreversible pathological change in the IPFP. Second, there was a potential limitation related to a technical problem: it was not confirmed whether there was no leakage of collagenase around the patellar tendon over time. Although injections were administered with great care, it is possible that the IPFP was injected by the needle. Third, the histological changes could not be evaluated in detail because these changes were assessed in only two histological examinations. To explain the elaborate pathogenesis, immunohistological evaluation or analysis of mRNA expression levels for molecules should be examined. Fourth, it could not be confirmed whether the rats in the PT group had symptoms such as abnormal gait and pain, or changes in tendon biomechanics. Lastly, PT was induced by the injection of chemicals. Although the use of mechanical overloading has an etiological advantage, intratendinous injection of chemicals has been used to establish tendinopathy animal models [14]. For an observational study, chemically induced PT may be helpful to assess the histology of the IPFP. By contrast, to facilitate the translation of research findings to clinical application for the management of exercise-induced tendinopathy in humans, the mechanical overloading model is more useful.

5. Conclusions

In conclusion, the IPFP in the group of rats with collagenase injected into their patellar tendon showed greater cellularity, fibrosis, and vascularity, and more severe fibrotic change than the controls. These inflammatory changes may also occur in humans. Being aware of these potential inflammatory changes may help physicians manage patients with PT.

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Declarations of interest

None.

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